DEVELOPMENT OF A PROTEIN MICROARRAY SYSTEM FOR THE DIAGNOSIS OF SEVERE EQUINE ASTHMA AND THE EFFECT OF FORAGE TREATMENT ON IgE-PROTEIN BINDING

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A thesis submitted to The University of Gloucestershire in accordance with the requirements of the degree of Doctor of Philosophy in the School of Equine Management and Science (Royal Agricultural University). This work was conducted in collaboration with University of Nottingham.

November 2019

Word Count: 36,241

Author's declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Gloucestershire and is original except where indicated by specific reference in the text. No part of the thesis has been submitted as part of any other academic award. The thesis has not been presented to any other education institution in the United Kingdom or overseas.

Any views expressed in the thesis are those of the author and in no way represent those of the University.

Signed:

Date: 27/11/2019

doi:10.46289/9S5W22HR

Acknowledgments

The work presented in this thesis was carried out at the Royal Agricultural University, School of Equine Science and Management, and the University of Nottingham, School of Biosciences, during the period of October 2015 to October 2018. Financial support was provided by the Fred and Marjorie Sainsbury Charitable Trust, the Royal Agricultural University and HAYGAIN, which I most gratefully acknowledge.

I would like to give a special thank you to Meriel Moore-Colyer, Professor of Equine Science, Royal Agricultural University, for her continued support. I wish to thank Marcos Alcocer, Associate Professor in Biochemistry, University of Nottingham, for his guidance and expertise. I also want to thank Prof Duncan Hannant, Dr Eric Richard, and the entire Alcocer group for their assistance and encouragement. Finally, I express sincere gratitude to my mother, for her endless support and always encouraging me to follow my dreams.

Furthermore, I would like to thank:

- Prof Eliane Marti
- Prof Vince Gerber
- Prof Laurent Couëtil
- Prof Jean Pierre-Lavoie
- Dr Emmanuelle van Erck

Abstract

Severe equine asthma (sEA) is a prevalent, performance limiting disease which occurs in genetically susceptible horses. Severe equine asthma is associated with increased levels of allergen specific immunoglobulin E (IgE), which along with intradermal and basophil activation tests, lead many to speculate the disease may be IgE mediated. Historically, a plethora of cumbersome and expensive techniques have been adopted to characterise antibody–allergen interaction, rendering widespread allergen assessment problematic. The aim of this thesis is to utilise recent advances in computational and robotic technologies to "print" hundreds of environmental allergens and monitor their reactions with specific immunoglobulins in sera/bronchoalveolar lavage fluid (BALF) from sEA/non-affected horses, and explore the effect of hay preparation methods on IgE-protein binding.

In paper I, conditions were optimised for printing, incubation, immunolabeling, biological fluid sources, concentration techniques, reproducibility and specificity. The developed platform identified novel allergens, an association between sEA and pollen sensitisation, and a positive correlation between sera/BALF specific IgE profile. Applying this technique with mathematical modeling, paper II demonstrated the high discriminatory power of this approach, enabling the accurate diagnosis of sEA using serological IgE as a biomarker. The platform identified environmental influences on IgE profiles, discrimination between differing IgE-mediated conditions, and revealed novel pollen, bacteria, mould and arthropod proteins associated with sEA. Latex proteins, a genus previously untested in the horse, were the most influential

variables. Further developing this platform into a competition technique, Paper III demonstrated the ability to identify sEA-associated allergens in hay samples, and assess the effect soaking and steaming have on IgE-protein binding using specific horse/hay combinations. Forage preparation techniques had no impact on hay allergenicity. This work provides a new, rapid and more accurate approach to sEA diagnosis, providing a platform for tailored management and the development of allergen-specific immunotherapy.

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1.0 List of Papers

This thesis is based on the following publications that will be referred to in the text by their respective Roman numerals:

Paper I - S. White, M. Moore-Colyer, E. Marti, L. Coüetil, D. Hannant, E.A. Richard and M. Alcocer. 2019. Development of a comprehensive protein microarray for immunoglobulin E profiling in horses with severe asthma. J Vet Intern Med. 2019;33:2327–2335. https://doi.org/10.1111/ jvim.15564

Paper II - S. White, M. Moore-Colyer, E. Marti, D. Hannant, V. Gerber, L. Coüetil, E.A. Richard and M. Alcocer. Antigen array for serological diagnosis and novel allergen identification in severe equine asthma. *Sci Rep* 9, 15170 (2019) doi:10.1038/s41598-019-51820-7

Paper III - S. White, M. Moore-Colyer, E. Marti, D. Hannant, E.A. Richard, E. van Erck and M. Alcocer. The effect of hay treatments on IgE-protein binding in horses with severe equine asthma via protein microarray profiling [*submitted*]

Conference proceedings resulting from this thesis:

White, S. 2019. Serological diagnosis of severe equine asthma: protein microarrays and mathematical modelling, in: Equine Immunology Workshop. Bern, Switzerland. [invited speaker]

White, S. 2019. Protein microarrays in equine asthma and allergies, in: International Symposium on Sports, Equine, Medical Science and Technology. Johor Bharu, Malaysia. [invited speaker]

White, S., Moore-Colyer, M., Marti, E., Coüetil, L., Hannant, D., Richard, E. and Alcocer, M., 2019. Microarray analysis and mathematical modelling in equine asthma diagnostics, in: European Academy of Allergy and Immunology Conference. Lisbon, Portugal.

White, S., Moore-Colyer, M., Marti, E., Coüetil, L., Hannant, D., Richard, E. and Alcocer, M., 2018. Diagnosis of severe equine asthma via protein microarray immunoglobulin E profiling and mathematical modelling, in: European Veterinary Immunology Workshop. Utrecht, Netherlands.

White, SJ., Moore-Colyer, MJS., Coüetil, L., Hannant, D., Richard, EA., Marti, E. and Alcocer, M., 2017. Development of a multiple protein extract microarray for profiling allergen-specific immunoglobulin E in horses with severe asthma, in: World Equine Airways Symposium. Copenhagen, Denmark.

2.0 Introduction

Severe equine asthma (sEA) has emerged as an important problem in the United Kingdom. Over 14% of horses are affected by this heterogeneous disease, which is associated with increased allergen specific IgE. Severe equine asthma is currently diagnosed based on history and clinical examination findings. Further tests often conducted include tracheal endoscopy, bronchoalveolar lavage, lung function test, thoracic radiographs and ultrasound. Bronchoalveolar lavage fluid (BALF) cytology can confirm lower airway inflammation through increased neutrophil counts, suggesting sEA. In horses whom do not respond to standard therapy, radiography is often utilised to further assess differentials. Less commonly, in vitro measurements of IgE (e.g. ELISA) or intradermal testing are utilised to establish allergens of interest in affected horses.

In fulminant cases, diagnosis can often be made based on history and clinical examination, however endoscopy and BALF cytology are commonly utilised to assist in classification. The collection of BALF is time consuming; requires the administration of prohibited substances (i.e. substances deemed by governing bodies to have a high potential for abuse, or no legitimate use in competition horses); is invasive; can trigger respiratory crisis, and is of little relevance in tailoring specific treatment. Therefore, the development of a clinically relevant test capable of diagnosing sEA via blood serum would be of huge benefit. A number of studies have documented a correlation between

allergen specific IgE and the likelihood of severe equine asthma. Several studies have suggested that sEA-affected horses have higher levels of specific IgE against certain environmental proteins, however due to the limited numbers of allergens included on the panel and the statistics employed for class prediction to date, it has not been possible to diagnose sEA through IgE analysis alone.

As knowledge of sEA inciting allergens and environmental exposure is so limited, it has been suggested that this may account for the inaccuracy and difficulties in utilising IgE as a biomarker in sEA diagnosis. IgE profiling and epitope mapping may become an additional tool for sEA diagnosis, treatment and potentially prediction. Furthermore, characterisation of allergens through IgE profiling would lead to better understanding of the aetiology and pathogenesis of this condition.

Previously, IgE profiling against specific allergens in the horse has been conducted using ELISA. Synthesis of large numbers of peptides, however, is fairly prone to error, time-consuming, labour-intensive, expensive, and has limitations due to the method's specific chemistry. A large quantity of serum is needed, and the number of targeted peptides is limited in this approach.

With the development of microarray technology and increasing availability of recombinant proteins, I have applied protein microarray-based immunoassays for allergen mapping of equine environmental allergens. There are numerous advantages: hundreds of target proteins can be tested concurrently using

small quantities of diluted serum, significantly reducing the biological sample requirements, cost of individual assays and enabling more robust replication and statistical approaches for analysing and determining allergens. Several immunoglobulin subclasses can be tested concurrently with IgE, allowing the simultaneous investigation of potential regulatory responses (e.g. IgA) that may influence clinical reactivity. However, a protein microarray platform that measures specific binding and gives reproducible results needs to be optimised and validated before it can be widely applied for allergen mapping of equine environmental allergens. This thesis describes the development and validation of a reliable protein microarray-based immunoassay based on proteins associated with the equine environment.

2.1 Severe equine asthma

Severe equine asthma is a performance limiting allergic response to airborne antigens present in organic dust, that effects approximately 111,440 horses in the UK (Bracher *et al.,* 1991; Halliwell *et al.,* 1993; McGorum *et al.,* 1998). The predominant source of these aeroallergens is hay, which contains high levels of potential sEA-associated allergens including pollen, mites, bacteria and mould (Bogacka and Jahnz-Rózyk, 2003; Künzle *et al.,* 2007; Moran and Folch, 2011; Pirie *et al.,* 2003; Tahon *et al.,* 2009). Exposure to these agents in sensitised horses results in mucus hypersecretion, bronchoconstriction and small airway inflammation; resulting in altered pleural pressure, pulmonary resistance and dynamic compliance (Miskovic *et al.,* 2007; Pirie, 2014).

Severe equine asthma has long been associated with mature horses, with the age of onset noted in horses over 7 years old, but more commonly between 9 – 12 years old (Couëtil et al., 2016; Couëtil and Ward, 2003; Hotchkiss et al., 2007a). Historically, the prevalence of sEA in Switzerland was considered to be 54.8% (Bracher et al., 1991b), however, more recent work has estimated the true prevalence of sEA in the northern hemisphere to be 10.7 – 17.4% (Hotchkiss et al., 2007a). Several risk factors have been associated with sEA, Couëtil and Ward (2003) demonstrated an increased risk of sEA with increasing age, with horses over the age of 7 years being 6 – 7 times more likely to be diagnosed with sEA. Furthermore, Thoroughbreds and ponies were 3 times more likely to be diagnosed; whereas horses were more likely to be diagnosed with sEA when examined in winter (1.6 times) and spring (1.5 times) (Couëtil and Ward, 2003). Further work utilising a logistic regression model, identified risk factors to include; increased age; exposure to an urbanised environment; exposure to hay in early life and respiratory infection in early life (Hotchkiss et al., 2007a). Conversely, multivariable modelling revealed that risk factors for geriatric horses differs to that of the general equine population, and includes being ridden daily (≥4 days/week), residing on a farm and having suffered with a respiratory infection in the preceding 12 months (Ireland et al., 2015).

Several studies have demonstrated a familial predisposition to this debilitating condition proving its genetic basis, however the mechanisms behind inheritance of sEA are still unclear. Gerber et al. (2008) revealed the risk of developing sEA is 3-fold increased when a single parent is affected and

increases to 5-fold where both dam and sire have sEA. A whole genome scan linked seven chromosomal regions with sEA, particularly chromosome 13 harbouring the IL4R gene was strongly linked (Gerber et al., 2008). The involvement of IL4RA as a candidate gene in sEA was further confirmed by Jost et al. (2007). Moreover, the quantity of IgE is influenced by hereditary factors in the horse, and sEA-affected offspring of the same stallion have increased levels of specific IgE against mould spore allergens (Gerber et al., 2008). Furthermore, it is well reported that the 'atopic march' in humans occurs as a sequential manifestation of hypersensitivities, in which dermatitis (atopic) often precedes the development of sEA (Bantz et al., 2014). Similarly, atopic dermatitis severity correlates with the risk of developing rhinitis, as well as increased quantities of both specific and total IgE (Gustafsson et al., 2000). Interestingly, these multiple hypersensitivities are also seen in horses, where it was demonstrated 13.1 times more likely for a sEA-affected horse to also be affected by insect bite hypersensitivity (IBH) (Kehrli et al., 2015). Kehrli et al. (2015) emphasised that there is an increased risk of horses suffering from sEA, urticaria or IBH to be affected by another hypersensitivity. Moreover, this predisposition to multiple hypersensitivities has been linked with reduced strongyle egg shedding, along with the polymorphism SNP BIEC2-224511 (singular nucleotide) (Gerber et al., 2015). This is particularly relevant as a genotypic association study found SNP BIEC2-224511 has a strong relationship with sEA in both unrelated and related horses (Shakhsi-Niaei et al., 2012). Furthermore, several previous studies have established that sEA is linked with strongyle parasite resistance in a related group of high-prevalence horses (Bründler et al., 2011; Neuhaus et al., 2010).

2.1.1 Aetiology of severe equine asthma

The inhalation of airborne particulate matter (APM) is an important cause of airway inflammation, and sEA is the most profound respiratory response to this; however even in healthy horses exposed to increased APM (in the form of organic particles), lower airway inflammation is induced (Wilsher et al., 2006). The World Health Organisation emphasises that the size of the APM plays an essential role in determining the effect that particles will have on respiratory health. The aerodynamic diameter of APM is split into the inhalable, thoracic (10 μm) and respirable (4 μm or smaller) fractions. The respirable fraction consists of particles small enough to penetrate the gas exchange regions of the lungs, which has previously been considered an appropriate indicator for assessing the risk to horse health in the stabling environment (Holcombe et al., 2006). However, others have suggested assessing APM of an aerodynamic diameter that are capable of penetrating the thoracic region may be a better measurement for assessing air quality in stabling (Holcombe et al., 2006; Sánchez et al., 2005; Widmer et al., 2009). Airborne particulate matter levels vary hugely depending on a multitude of factors including; stable design, management, ventilation and activity (Ivester et al., 2014). APM measurements may be conducted using two main methods 1) a personal sampler placed in the breathing zone of the horse or 2) a stationary air sampler. Both of these methods have a variety of merits and limitations. Personal sampling often consists of an air pump to which tygone

tubing and a filter cassette is attached near the nostril of the horse allowing for effective *in situ* sampling of the true APM exposure challenge. However, this is time consuming, poses safety issues and the results are variable, depending on the activities of the specific horse to which it is attached (Clements and Pirie, 2007). Static air samplers negate these limitations, and devices (such as Dustrax) enable real-time monitoring, but must be situated in a location where it is possible to estimate true exposure or account for the self-generated particulate from specific activities such as consuming forage (Millerick-May et al., 2011). Airborne particulate matter levels can significantly differ with seasonality, as Millerick-May et al. (2011) demonstrated that average APM concentration was at is highest in November and September, while in July it was at its lowest; full day APM mapping also revealed significantly higher concentrations during the early morning; stables with little ventilation had increased APM; and APM between 0.5 - 5 μm were lowest in November. Moreover, the location of the stable within the barn also negatively affected APM levels.

The factors that affect air quality influence the athletic performance and welfare of the animal (Clarke, 1987). Fine particulate are able to enter the pulmonary alveoli and constitute 30 – 40% of the total quantity of APM in the stabling environment (Crichlow et al., 1980). The predominate portion of these respirable particulates are associated with thermophilic and thermotolerant actinomycetes, and fungi (Clarke and Madelin, 1987). Mouldy hay challenge in horses with sEA has been shown to result in changes in clinical score, breathing rate, work of breathing, peak tidal inspiratory, arterial O_2 and CO_2

tensions, expiratory flow rates, dynamic compliance and lung resistance (Tesarowski et al., 1996). Poor meteorological conditions during hay harvesting in the United Kingdom often results in baled hay with a moisture content in excess of 20%, which increases the proliferation of potential sEA inciting allergens, including bacteria, endotoxins, mites, mould and their metabolites (Seguin et al., 2010; Séguin et al., 2012). When comparing two management systems, Woods et al. (1993) demonstrated that use of hay and straw compared to pelleted diets and wood shavings resulted in substantially higher levels of respirable particles (97%), including *Aspergillus fumigatus* (1823 ng/m3 and 748 ng/m3), *Sacharopolyspora rectivirgula* (1423 ng/m3 and 705 ng/m3), and mite allergens (1420 ng/m3 and 761 ng/m3) (Woods et al., 1993). More recently, the influence of hay and bedding contributing viable fungal spores or endotoxins within the stabling environment was emphasisied, as well as, demonstrating that negative air-ionizers do not significantly reduce the levels of dust (Siegers et al., 2018). In the stabling environment, hay is the major source of these aeroallergens (Webster et al., 1987).

Although much attention is given to APM concentrations in the stabling environment, little thought is given to the riding arena. Qualitative mould studies demonstrate mostly *Aspergillus spp., Cladosporium spp.* and *Penicillium spp.* are present in the APM in riding arenas. The APM concentration and airborne mould concentration can vary depending on a variety of factors, including; footing material, season, moisture content, activity (before/during), maintenance, number of horses, gait and location of the arena (Rapp et al., 1992). Total dust level with inorganic footing (0.706 mg m(-3))

and organic footing (0.600 mg m(-3)), as well as, respirable dust with inorganic footing (0.429 mg m(-3)) and organic footing (0.327 mg m(-3)) were both lower than levels widely purported in the stabling environment (Wheeler et al., 2006). Interestingly, the quantities of APM detected correlated more so with the quality of artificial surface laid in the arena, as oppose to dust suppression methods (e.g. watering). Airborne particulate matter 0.3-0.5 μm was lowest in a woodchip arena compared to that consisting of just sand (Lühe et al., 2017); however, woodchip poses a greater source of airborne moulds (Rapp et al., 1992). The lowest concentration of airborne mould was detected after the arena had not been used overnight (10 hours) (Claußen and Hessel, 2017).

These pathophysical changes appear to be reversible when the sEA-affected horse is removed from an average stabling environment. When symptomatic horses are placed in a low-respirable particle environment (i.e. shavings and pelleted diet), they become asymptomatic within approximately 8 days, seeing their pulmonary function values becoming comparable with control horses (Thomson and McPherson, 1984). Similarly, pulmonary function tests revealed sEA-affected horses at pasture, or in an allergen-avoidance stabling program were comparable with healthy horses (Kirschvink et al., 2002). Furthermore, sEA horses in contact with hay in a barn environment develop clinical signs within approximately 8 days and show significant alteration in mechanics of breathing and arterial bloods (Vandenput et al., 1998a), but then show parameters similar to healthy horses when at pasture or fed silage in a stabling environment (Vandenput et al., 1998b). More recently, Miskovic et al. (2007) demonstrated sEA-affected equids in clinical remission within a low

dust environment may still have low-grade inflammation due to irreversible airway remodelling.

Several methods have been used to assess which allergens may be implicated in the aetiology of sEA, including inhalation challenge, intradermal testing and specific immunoglobulin E (IgE) analysis. Inhalation challenges with extracts of *Thermoactinomyces vulgaris*, *Saccharopolyspora rectivirgula*, *Aspergillus fumigatus*, and hay dust, confirmed the roles of *S. rectivirgula*, *A. fumigatus* and hay dust in the aetiology of sEA (clinical signs, BALF cytology, arterial blood gas tension/pH, and pulmonary mechanics) (McGorum et al., 1993a). Further work in *S. rectivirgula* extract inhalation demonstrated its role in sEA, by inducing pulmonary neutrophilia, abnormalities of ventilation (Derksen et al., 1987), increasing pulmonary resistance and respiratory frequency minute ventilation, as well as, decreasing arterial oxygen tension (Derksen et al., 1988).

Inhalation challenges with lipopolysaccharides (LPS) contributes to airway inflammation in conjunction with other inhalants in sEA horses and in healthy horses stabled in a poor environment (Pirie et al., 2001). Hay dust inhalation challenge in sEA horses results in mucus hypersecretion, obstructive airway dysfunction and airway neutrophilia, and the severity of this response appears to be dependent on dose (Pirie et al., 2002). Further work by Pirie et al. (2003) confirmed the aetiology of *A. fumigatus* in sEA, and demonstrated LPS amplified horses response when inhaled with A. *fumigatus*, suggesting it may play a greater role in sEA than first thought. More recently, *Lichtheimia*

corymbifera, *A. fumigatus*, and *Eurotium amstelodami* fungal spores in combination with lipopolysaccharide, and microspheres were shown to induce an increase in transpleural pressure and airway neutrophilia in sEA-affected horses (Beeler-Marfisi et al., 2010).

As sEA has previously been associated with immediate hypersensitivity reaction, intradermal testing has been utilised to assess inciting allergens. Initial tests using *S. rectivirgula*, proved to be accurate in diagnosing sEA, unlike *A. fumigatus* extracts (McPherson et al., 1979). McGorum et al (1993) demonstrated dermal and pulmonary reactivities did not correlate using extracts of *S*. rectivirgula*, T. vulgaris,* and *A. fumigatus* in the horse, therefore indicating the use of intradermal testing using mould and bacterial extracts is of little diagnostic relevance. Wong et al. (2005) found reactions with intradermal mixed aspergillus extract was only detected in sEA-affected horses. More recent studies have also found little efficacy in intradermal testing for the diagnosis of sEA, but suggested further studies were required into the clinical relevance of mite allergen and rAsp f 8 (Tahon et al., 2009). Lorch et al. (2001) compared the results of intradermal testing with serological ELISA results, demonstrating a positive correlation. Similarly, more resent research assessing both bronchoalveolar lavage fluid (BALF) and serum IgE with intradermal testing, found no correlation between results, but found intradermal testing to agree with Lorch et al. (Tilley et al., 2012).

The most common method for assessing inciting allergen involved in the aetiology of sEA is via specific IgE (sIgE) analysis of either of BALF or serum.

Halliwell et al. (1993) noted higher levels of BALF sIgE recognising *S. rectivirgula* and *A. fumigatus* extracts in sEA-affected horses, but no differences with serum analysis using ELISA methods. A similar study investigating *A. fumigatus* found no difference between affected and control horses in correlation between BALF and serum, but reported elevated levels of BALF sIgE of affected horses recognising 93, 35, 31 and 23 kDa allergens (Schmallenbach et al., 1998). Using serological ELISA methods Eder et al. (2000) demonstrated sEA horses are more likely to be sensitised to *Alternaria alternate* and *A. fumigatus* than non-affected horses, while emphasising that recombinant proteins enable increased sensitivity in serological specific immunoglobulin isotype profiling. Further serological ELISA studies assessing *A. fumigatus, A. alternate* and rAsp f 8, found sIgE levels to be higher in sEA horses, particularly those recognising rAsp f 8 (Künzle et al., 2007). Another serological ELISA study concluded sEA is associated with increased sIgE against mites, in particular *T. putrescentia* (Niedzwiedz et al., 2015). Using western blot methods to evaluate BALF, Couetil et al. (2015) found increased sIgE against *Alternaria alternata, Wallemia sebi, Eurotium amstelodami* and *Aspergillus terreus* in sEA-affected horses.

Dirscherl et al. (1993) used an *in vitro* basophil assay to evaluate inciting-allergen associated with sEA, and concluded sensitivity of the basophils of affected horses was increased against the allergen extracts of *Mucor* (*Mucor mucedo and Mucor spinosus*), *Penicillium (P. brevicompactum, P. expansum, P. notatum)* and *Cladosporium herbarum*.

It is acknowledged that a wide variety of potential allergen, including pollens, are yet to be elucidated, but due to the limited availability of recombinant proteins, as well as, the limitations of classic cumbersome techniques, such as ELISA, only a limited panel have been screened to date.

2.1.2 Pathogenesis of severe equine asthma

The pathogenesis of sEA remains unclear; several studies have implicated the role of IgE, suggesting these IgE antibodies mediate a type I hypersensitivity leading to sEA (Moran et al., 2010a,b; Kunzle et al., 2007; Curik et al., 2003; Eder et al., 2000; Schmallenback et al., 1998; Halliwell et al., 2003). In this context, the cross-linking of basophil/mast cell-bound IgE antibodies by specific proteins represents the initial signal for the release of inflammatory mediators, resulting in associated symptoms (see figure 1).

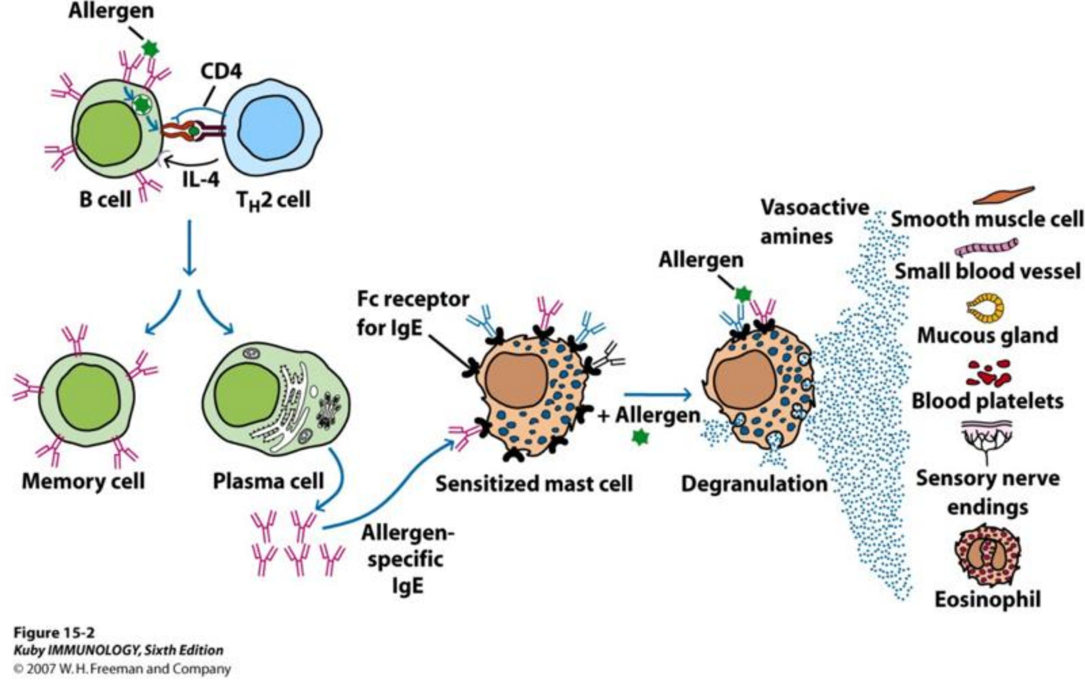

Figure 1: Hypersensitivity type I

Several methods have been utilised to identify allergens associated with the pathogenesis of sEA, such as *in vitro* stimulation of pulmonary mast cells, blood basophils and RBL-2H3 (basophil-derived cell line), adding weight to this type I hypersensitivity theory (Dirscherl et al., 1993; Hare et al., 1998, 1999a; Moran et al., 2012; Morán et al., 2010a, 2010c). Moreover, Gerber (1989) demonstrated there was an increased risk of sEA in the offspring of affected horses, with a substantial effect on both IgE recognising recombinant *A. fumigatus*, as well as, total IgE levels, evidencing the effect of genetics on sIgE. As with human asthma, it is though that sEA is a heterogeneous disease, and therefore not all cases may IgE-mediated, however an association between sEA and sIgE can be seen.

Asthma in humans is characterised by the expression of several genes specific for inflammatory proteins, such as cytokines, adhesion molecules, receptors and enzymes. This process is primarily driven by transcription factors, these proteins can bind to the promoter region of the aforementioned genes, and can be activated by specific inflammatory mediators (e.g. cytokines). Moreover, asthmatic inflammation also involves a plethora of transcription factors, such as: signal transduction-activated transcription factors (STAT), cyclic AMP response element binding protein (CREB), nuclear factor of activated T-cells (NF-AT), activator protein-1 (AP-1), and nuclear factor-kappaB (NF-kB). Working in tangent, several inflammatory genes are produced as a result of these transcriptions (Barnes and Adcock, 1998). Similarly, the airway epithelial cells of sEA-affected horses in contact with

mouldy hay show raised expression of NF-kB, most notably the p65 homodimers, and increased AP-1 binding (Bureau et al., 2000a,b; Saunders et al., 2001). However, continued exposure to the offending allergen results in upregulation of CREB and down regulation of AP-1 (Couetil et al., 2006). These findings suggest that the transcription factors CREB, AP-1 and NF-kB, are all crucial elements in modulating airway inflammation in sEA-affected horses.

A crucial role is played by T cells in modulating immunological responses in the pathogenesis of sEA. More recently, it has been suggested that pulmonary T helper (Th) cells, through the secretion of Th 1 or Th 2 cytokines, could have a substantial involvement in the pathogenesis of sEA (Riihimaki et al., 2008; Ainsworth et al., 2007; Ainsworth et al., 2003; Cordeau et al., 2004; Giguere et al., 2002; Lavoie et al., 2001). Depending on the time of collection and stage of disease, sEA-affected horses produce both Th 1 and Th 2 cytokines. Moreover, subsequent studies have confirmed this Th2 response within lungs of both horses with sEA and human asthma patients (Pacholewska et al., 2015), demonstrating that this Th 2 response is crucial in recruiting and activating IgE producing B cells, eosinophils and mast cells involved in type I hypersensitivity (Deo et al., 2010). Th2 cells have been well documented in sEA-affected horses. Cordeau et al. (2004) found increased mRNA for IL-4 and IL-5 being expressed by lymphocytes, concluding that throughout clinical exacerbation, there is predominantly a Th 2 cytokine response which occurs in tandem with airway obstruction. Dewachi et al. (2006) found asymptomatic sEA horses that have been at pasture for 3 months showed higher levels of neutrophil expressed IL-5 and IL-9 receptors in comparison to non-affected

horses at pasture, which increased further when stabled, further indicating that a Th 2 cytokine profile is associated with sEA, as seen in human allergic asthma, further supporting the hypothesis that both equine and human asthma share a similar pathogenesis (Lavoie et al., 2001). Conversely, Horohov et al. (2005) suggested sEA affected horses express a modified Th 2 cytokine profile characterised by the expression of IL-13 and IL-4, but not IL-5 in BALF. Furthermore, sEA-affected horses in contact with mouldy hay expressed higher levels of TNF-alpha and IL-1 beta, as well as IL-8 mRNA expression being higher in affected horses (Giguère et al., 2002). Moreover, increased IL-5 and IL-4 mRNA and decreased IFN-gamma expression has been reported in sEA-affected horses (Lavoie et al., 2001). Furthermore, Wagner (2009) suggested that the literature does not support the IgE-mediated pathogenesis of sEA, noting it is more characteristic of hypersensitivity type IV, and suggested this has been confirmed by both Ainsworth et al. (2003) and Giguère et al. (2002) whom both demonstrated increased IL-8 and IFN-gamma expression in sEA-affected horses. Moreover, the inflammatory response is characterised by bronchial neutrophilia which is potentially indicative of a type III hypersensitivity response. In this scenario it is suggested inflammation occurs due to complex formations of antigen-antibody complexes which results in the activation of the complement cascade resulting in anaphylatoxin peptides C3a and C5a release (Lavoie et al., 2001). These affect the contraction of smooth muscle, as well as enhancing vascular permeability; moreover, they recruit a range of leukocytes, thus signalling neutrophilic and eosinophilic oxidase activities and subsequently leukocyte degranulation. The Th2 type cytokine provides a possible explanation to how

neutrophils are activated in the equine respiratory immune response. Although the factors that lead to the initiation of airway neutrophilia in sEA-affected horses is unelucidated; it is well understood that an intraluminal accumulation of neutrophils will occur within hours of an environmental challenge (Fairbairn et al., 1993). Increases of IL-8 in BALF and BALF cells has been identified in the sEA affected horse alongside an increase in neutrophils, post challenge (Ainsworth et al., 2003; Giguere et al., 2002). Bronchial NF-kB is initiated by both nongranulocytic and granulocytic cells, furthermore the levels of bronchial NF-kB correlates with the levels of neutrophils present (Bureau et al., 2000a). Furthermore, much attention has been given to IL-17 as this interleukin induces proinflammatory cytokines, including IL-1B, IL-6 and TNF-α, similarly the chemokines CXCL1, 2, and 8, thus inflammation and neutrophilia (Schmidt-Weber et al., 2007). However, Riihimaki et al. (2008) demonstrated an increased IL-17 expression is proceeded by increased neutrophils in the pulmonary tissue; suggesting IL-17 may not contribute to neutrophilia, but rather have a role in maintenance of inflammation in sEA-affected horses. Moreover, other researchers have suggested that cytokines alone may be responsible for the mucus hypersecretion, neutrophilia and altered airway functions readily associated with sEA (Debrue et al., 2005). Furthermore, MUC5AC expression, which initiates the hypersecretion of mucus, may be stimulated by IL-17 (Gerber et al., 2003).

Despite the likely heterogeneity in immunological pathways leading to sEA, several studies have evaluated the role of IgE in the pathogenesis of this disease. Some studies have rejected this IgE-mediated theory (Marti et al., 2008; Wagner, 2009), predominantly due to the fact delayed-type

hypersensitivity appears to be involved. However, a plethora of studies have supported the IgE-mediated pathogenesis of sEA (Moran et al., 2010a,b; Kunzle et al., 2007; Curik et al., 2003; Eder et al., 2000; Schmallenbach et al., 1998; Halliwell et al., 2003). The contradiction in these studies further goes to confirm the heterogeneous nature of sEA.

2.1.3 Clinical signs of severe equine asthma

Common clinical signs associated with sEA include: increased respiratory effort, nasal discharge, exercise intolerance and chronic coughing (McGorum et al., 1993a). These signs will usually occur in horses over 7 years old, on exposure to aeroallergen. There may be a familial history of the disease, and it may occur seasonally (Couëtil et al., 2016). The most commonly associated clinical complaint is coughing, which can range in frequency, and is considered chronic if it persists for over 1 month (Derksen et al., 1989). In a retrospective study, it was confirmed 71% of referred sEA cases had coughed for 3 or more months (McPherson et al., 1979). Although bilateral nasal discharge, often mucopurulent in nature, is commonly associated as a clinical sign of sEA, in a retrospective study it was demonstrated that only 50.4% of referred cases suffered with nasal discharge on the week proceeding assessment (Dixon et al., 1995). Exercise intolerance is reported in over half of cases, presenting as poor recovery rate, prolonged increase in respiratory rate and dyspnoea following cessation of exercise. The increased respiratory rate is often a result of compensation for impaired lung function. This rate will often remain high for a long-time post-exercise in sEA affected horses (Muylle and Oyaert, 1973).

This is due to pulmonary hypertension associated with systemic arterial hypoxia (Dixon, 1978, Benamou et al*.,* 1998). Furthermore, almost two thirds of sEA horses will display a “heaves line” resulting from the increased use of abdominal muscles during exhalation causing abnormal musculature (Aviza et at., 2001). Flared nostrils are another common sign, a result of trying to reduce resistance due to the increase in effort needed to breath (Naylor et al., 1992, Robinson et al., 2000). Neutrophilic inflammation occurs in the airways within several hours of inhaling hay dust fraction (Fairbairn et al., 1993). Reversibility of clinical signs is a major characteristic of sEA, and should occur when the horse is turned out or in a low dust environment.

2.1.4 Diagnosis of severe equine asthma

Diagnosis is possible purely on clinical signs, as demonstrated by Laumen et al. (2010). However, several ancillary tests are often performed to confirm diagnosis.

Airway endoscopy is performed to assess tracheal mucus accumulation in the tracheobronchial tree, endoscopic mucus grades above 2 out of 5 may indicate sEA (Couëtil et al., 2016). Endoscopic scoring of tracheal sputum thickness is commonly used in the human for evaluating lower airway disease, but is yet to be assessed in the horse.

Airway cytology is one of the most common diagnostic assessments for sEA. Tracheal wash (TW) cytology contains several biomarkers pertaining to immune function of the lower airways (Hoffman, 2008). BALF remains the gold-standard for cytological analysis and remains the predominant assessment for sEA-associated inflammation. Interestingly, there is no correlation between tracheal and BAL cytological profiles as BALF better reflects the cellular response of lung injury (Derksen et al., 1989), furthermore Hoffman (2008), stated there are many advantages to BALF over TW cytology, as BALF correlates well with the clinical signs associated with sEA. Bronchoalveolar lavage fluid cytology profiles from sEA-affected horses usually show moderate - severe neutrophilia (>25% cells), as well as decreased ratios of both alveolar macrophages and lymphocytes (Couëtil et al., 2001; Derksen et al., 1985).

Many different methods may be used to assess lung function. Traditionally transplural pressure evaluation allows for calculation of work of breathing, dynamic compliance and lung resistance. Pleural pressure measurements correlate with pulmonary resistance and dynamic compliance, ergo are an accurate indicator of sEA through altered breathing strategies. More recently, oesophageal balloon catheter techniques have been recommended for assessing moderate - severe airflow limitation during disease exacerbation (DPmax >15 cm H_2O) (Couëtil et al., 2016). Arterial blood gas analysis was once commonly used to assess decreases in PaO_2, but is less commonly used now due to its lack of sensitivity.

Airway hyper responsiveness has previously been utilised as a diagnostic method where a dose of histamine is given and changes in lung mechanics, indicative of bronchoconstriction, are used to quantify airway reactivity (Mazan et al., 1999). Similarly, the reversibility of sEA with a bronchodilator or environmental change, is considered to support diagnosis.

Several studies have assessed allergen-specific assays in the diagnosis of sEA. To date, intradermal testing, histamine release assays and enzyme immunoassays have all yielded conflicting results, and have proven to be of poor diagnostic value to sEA (Lorch et al., 2001; McGorum et al., 1993b; Woods et al., 1993) . Ergo, these types of allergen-specific diagnostic approaches require further research to enable future use within the veterinary diagnostics sector, but all researchers agree this technique would have many advantages in both diagnosis, treatment and patient management.

2.1.5 Treatments for severe equine asthma

Although the most important change that can be made in treating horses with sEA is allergen-avoidance (Robinson, 2001) several anti-inflammatory medicines are used orally, intravenously or via inhalation, to provide short-term relief.

Corticosteroid therapy, commonly prednisolone and dexamethasone, is often given orally due to its ease of administration, however parenteral

administration is more effective. Moreover, the addition of an oral corticosteroid (prednisolone) is often administered initially, for quick enhancement of airway functionality, but this does not improve the clinical response which resulted from modifying the environment (Jackson et al., 2000). Furthermore, corticosteroids are also useful when allergen avoidance is not possible, as both prednisolone and dexamethasone enhance pulmonary functionality, in spite of continued exposure to allergen/antigen, dexamethasone being most effective (Leclere et al., 2010). Historically, the administration of corticosteroids has been associated with laminitis, although, more recent studies have contested this (Cornelisse and Robinson, 2013; Potter et al., 2019).

Bronchodilators, such as the β_2 adrenergic agonist clenbuterol is commonly utilised in sEA-affected horses for its renowned effects as a bronchodilator. These bronchodilators are most commonly given orally (e.g. Ventipulmin Syrup) (Erichsen et al., 1994). Clenbuterol also has an extra anti-inflammatory effect, significantly decreasing the expression of a plethora of proinflammatory cytokines and chemokines and increasing the expression of IL-6 (Laan et al., 2006a). Other bronchodilators include aerosolised albuterol, which proves effective in horses with sEA (Bertin et al., 2011), and also treats bronchospasm (Derksen et al., 1999). Furthermore, undesirable side effects have been noted with aerosol pirbuterol despite its efficacy as a bronchodilator (Derksen et al., 1992). Derksen et al. (1996) concluded aerosol pirbuterol was desirable as it provides fast acting alleviation of symptoms, as well as the overall significance of drug effect and lack of side effects. More recently,

trimetoquinol has been used intravenously as a fast acting bronchodilator ideal for administration during crisis (Camargo et al., 2006), and also proved to be just as effective when administered as an aerosol (Camargo et al., 2007).

Long-term use of bronchodilators and corticosteroids is often not possible due to increased likelihood of drug-associated adverse effects and concerns regarding drug residues when competing under FEI and Jockey-Club rules, therefore environmental management is the cornerstone to effective treatment of sEA (Couëtil et al., 2005). Allergen avoidance alone improves lung function within 3 days, emphasising its importance in sEA treatment. Improvement continued until day 7, with the best function coming after 30 days at pasture, even when compared with medical intervention (prednisone), emphasising the importance of allergen avoidance in sEA treatment (Jackson et al., 2000). Airborne dust concentration and sEA-associated allergen (via radioallergosorbent-inhibition immunoassay) was measured in 2 differing stabling environments: 1) hay and straw bedding (HS) and 2) wood shavings and a complete pelleted diet (WP). Results showed, dust concentrations were over three times higher in HS (Jackson et al., 2000). Suspected allergen exposures were also substantially increased in the HS environment when compared with WP: mite allergens (1420 ng/m3 and 761 ng/m3), *Aspergillus fumigatus* (1823 ng/m3 and 748 ng/m3), and *Saccropolyspora rectivirgula* (1423 ng/m3 and 705 ng/m3). Moreover, Seedorf et al. (2007) demonstrated that biocompost was inappropriate as a bedding material for horses due to increased concentrations of thermophilic actinomycetes and further potentially sEA inciting antigen. Wood shavings from the Pinacea family have been

shown to inhibit bacterial growth and are therefore highly recommended (Yarnell et al., 2017). Researchers found minor alterations between paper, wood shavings and straw in clean stables when the bedding was well managed, but did indicate that hay is a significant source of respirable particulate (Webster et al., 1987). More recently, an investigation into the effect of different stable designs on APM revealed steamed hay and shavings to be most effective in reducing APM, and emphasised the importance of avoiding straw and/or dry hay as an appropriate management protocol for stabled horses (Auger and Moore-Colyer, 2017). Despite this, hay is a necessity for many, and is fed by 55% of owners (Hotchkiss et al., 2007; King, 2012; Robinson, 2001; Vandenput et al., 1997). Poor weather conditions during hay harvesting results in organism proliferation, although, limited numbers of specific mould and bacteria strains have been identified in hay, with many researchers testing for genus, or total colony values (Clarke, 1987; Madelin et al., 1991; Moore-Colyer et al., 2014; Seguin et al., 2010; Séguin et al., 2012). Historically, owners of sEA-affected horses have relied on soaking hay to reduce respirable particle numbers, thus reducing the quantity of aeroallergens. However, recent studies have found soaking hay increases bacteria up to five-fold (Moore-Colyer et al., 2014), which could potentially be counterproductive in treating sEA, as hypothetically non-viable organisms and ingested spores could still precipitate an allergic reaction in sensitised horses (Tizard, 1977). Recent work in hay preparation methods revealed steaming hay reduces bacteria (99%), mould (99%) and respirable particle count (94%), indicating it may be beneficial in reducing fodder allergenicity (Moore-Colyer et al., 2015; Moore-Colyer et al., 2014; Stockdale and Moore-Colyer 2010).

Furthermore, Blumerich et al. (2012) found feeding steamed hay to sEA-affected horses reduced the characteristics of sEA (clinical score and total airway neutrophilia). Work is required on the most effective treatment of forage to reduce aeroallergens in the breathing zone of the horse and thus be able to make recommendations on the best method for treating fodder to reduce sEA in horses.

2.2 IgE as a biomarker in equine allergies and asthma

2.2.1 IgE overview

Equine asthma and other allergies represent prevalent and often performance limiting inflammatory responses to environmental allergen. In general, many allergies involving food and environmental allergens are classified as hypersensitivity type I, i.e., involve allergen-specific IgE. In this context, the cross-linking of basophil/mast cell-bound IgE antibodies by specific proteins represents the initial signal for the release of inflammatory mediators, resulting in associated symptoms. This reaction takes place in those that are genetically predisposed, and such horses have specific IgE (sIgE) in their serum indicating past exposure and sensitisation to that allergen (Johansson et al., 2004). As such specific sIgE has been used as a biomarker for the diagnosis of several allergies and inflammatory diseases, as well as elucidating aetiological factors (Deinhofer et al., 2004).

Immunoglobulins consist of both heavy and light chain loci, which are made up of gene products. The genes present in the constant region of the heavy

chain are responsible for determining the immunoglobulin isotype. The heavy chain constant region in horses is located on chromosome 24 (ECA24qter). A single immunoglobulin heavy constant epsilon (IGHE) gene encodes the IgE heavy chain constant region between the immunoglobulin heavy constant gamma (IGHG)5 and immunoglobulin heavy constant alpha (IGHA) genes. In contrast to humans, IGHE pseudogenes are not present in equids. Similarly, to other mammals, this IGHE gene consists of 4 exons, all of which encode a singular immunoglobulin domain present on the heavy chain region of IgE. The IGHE genes nucleotide sequences have been investigated by several research groups, revealing at least 4 separate IGHE alleles are present in equids, and these allele nucleotide sequences vary between 95.9-99.8% homology. Many of these nucleotide sequences also resulted in varying amino acids in the constant heavy chain region of IgE. It is not yet known if this allele variation of the IGHE gene has any influence on the horse's immune response to either allergies or parasites.

Six N-glycosylation sites are present on the constant heavy chain region of IgE, a particularly noteworthy site at position 174 which is not maintained in the allotypes of IgE*03. At position 270, the N-glycosylation is conserved between mammalian species. In humans, IgE binding and N-glycans are not directly linked, however, the N-glycosylation site 270 is essential in the function and formation of IgE molecules by enabling normal folding essential for binding in IgE receptors. In the horse, expression of IGHE*02 gene as part of a 4-(hydroxyl-3-nitro-phenyl)acetyl (NP) specific equi-murine recombinant

antibody in mammalian cells created a substantially glycosylated IgE chimera, which in turn mediated both *in vivo* and *in vitro* histamine release.

IgE is detectible in human serum, and is found at concentrations down to the nanogram per millilitre. IgE has a half-life of approximately 2 days, which is relatively short, this is a result of its binding to the alpha chain of the high-affinity IgE receptor (FcεRI) on basophil and mast cells when out of circulation. In recent years the creation of immunoassays for total IgE detection in horses has been facilitated by the production of monoclonal antibodies (mAbs) (Wilson et al., 2006). IgE levels are notoriously lower than other immunoglobulin isotypes in most mammalian species, and as such has added complexities to sIgE assessments in humans (Amarasekera, 2011). Interestingly, total serological IgE levels of the healthy horse are noted to be approximately 1000-fold higher than that of the human, potentially a result of the higher parasitic load in horses when compared to humans (Wagner, 2009). Similarly, serological total IgE levels of horses with insect bite hypersensitivity (IBH) was significantly higher than that of healthy horses, at 108.9 ± 69.0 ug/ml and 84.0 ± 90.9 ug/ml, respectively (Wagner et al., 2003). Moreover, horses kept in varying environments show variable IgE levels (Eder et al., 2000a), similarly, Wilson et al., (2006) demonstrating a significant IgE variations between horse breeds, when investigating IBH-associated IgE profiles differences were established between healthy Icelandic horses and IBH-affected horses, but no significant difference between affected and non-affected horses of other breeds.

The presence of IgE+ cells in lung tissue has been demonstrated via immunochemistry utilising anti-IgE (polyclonal) (Wagner et al., 2009). Furthermore, IgE+ mast cells were identified in non-affected horses, where immediate skin reactions resulted while using monoclonal anti-IgE 134 in intradermal skin trials. However, different monoclonal antibodies were not able to induce an immediate skin reaction, including anti-IgE 176. Despite this, horse anti-IgE mAb 134 and 176 IgE+ cell staining did not significantly differ in peripheral blood cells. Both of the mAbs have been shown to recognise different IgE heavy chain constant region epitopes (Eckl-Dorma et al., 2019; Wagner et al., 2003). Results from *in vitro* mediator release and intradermal assessments indicated that anti-IgE 134 induces IgE crosslinking and subsequent signalling of the high-affinity IgE receptor alpha chain causing basophil and cell mediator release, whereas anti-IgE 176 does not crosslink. Despite this, further work in this area is required for increased insight into mast cell and basophil IgE receptor expression and their role in the immune response. Work by Wagner et al. (2009) developing mAbs, such as those to equine CD23 and alpha chain of the high-affinity IgE receptor (alpha) enable further understanding of mast cell IgE receptor expression in horses.

2.2.2 The association between sIgE and sEA

As discussed in section 2.1.2, several studies have found an association between increased sIgE and/or *in vitro* stimulation of pulmonary mast cells/blood basophils, and sEA (Dirscherl et al., 1993; Hare et al., 1998,

1999a; Moran et al., 2012; Morán et al., 2010a, 2010c). *In vitro* basophil and pulmonary mast cell assays have been used to identify *Alternaria tenuis*, *Aspergillus fumigatus* and *saccharopolyspora rectivirgula* as potentially inciting allergens in sEA (Dirscherl et al., 1993; Hare et al., 1999b, 1998; Moran et al., 2012; Morán et al., 2010b, 2010c). Intradermal testing in horses with sEA revealed reactions to *A. fumigatus, S. rectivirgula,* mite allergen and rAsp f 8 (Wong et al., 2005; McGorum et al., 1993; McPherson et al., 1979; Tahon et al., 2009). ELISA methods have also been used to asses sIgE analysis in BALF, identifying reactions to *S. rectivirgula* and *A. fumigatus* (Halliwell et al., 1993b; Schmallenbach et al., 1998), and with sera, establishing reactions to *A. fumigatus, Alternaria alternate*, rAsp f 8 and mites, in particular *T. putrescentia* (Eder et al., 2000a; Künzle et al., 2007; Niedzwiedz et al., 2015). Using western blot methods to evaluate BALF, Couetil et al. (2015) found increased sIgE against *Alternaria alternate, Wallemia sebi, Eurotium amstelodami* and *Aspergillus terreus* in sEA-affected horses. Although the association between sEA and these various allergens has been ascertained, the limited range of allergens screened to date, and statistical approaches have prevented diagnosis with this biomarker.

2.2.3 Anti-Horse IgE Antibodies

Antibodies to equine IgE have been produced by several groups using a variety of methods. Several researchers have produced polyclonal anti-horse IgE using purified equine serological IgE for immunisation, but high amounts

of IgG isotype contamination is a major limitation of this preparation technique and can alter IgE results (Halliwell and Hines, 1985; Suter and Fey, 1981). Other groups attempted to increase specificity, producing polyclonal antibodies by immunising with E coli expressed recombinant partial IgE heavy chain regions (Marti et al., 1997). However, the bacteria expressed IgE may have limitations as glycosylation does not occur in these methods, which is crucial for the constant heavy chain region. Major limitations with polyclonal anti-IgE antibodies include their limited availability, variability between batches and increased potential of cross reactivity.

To combat this, several groups have developed anti-horse IgE monoclonal antibodies. Wagner et al. (2003) produced mAbs by expressing recombinant IgE in mammalian cells for immunising mice. More recently, Wilson et al. (2006) produced mAbs immunisation protocol involving recombinant bacterial and mammalian expressed IgE heavy chain constant regions and purified equine serological IgE. Comparisons between these different mAbs revealed their efficacy to be comparable using both ELISA and immunohistochemistry (Wagner, 2009).

2.2.4 Immunoassays for specific IgE

Several test methods may be used to establish sensitisation with IgE to specific allergen. These include *in vivo* analysis via intradermal testing, as well

as, *in vitro* histamine release assays and sIgE profiling using biological fluids, most commonly serum.

Intradermal testing relies on the injection of a small amount of the chosen allergen, and observing the physical reaction occurring due to the release of proinflammatory mediators, which will manifest as a wheal within 30 minutes (Wagner et al., 2006). The relevance of intradermal testing in sEA is controversial, with contradictory reports on its ability to identify allergens of interest (Tahon et al., 2009). Further studies are required to establish the clinical relevance of intradermal testing in sEA-affected horses, and its correlation with immunoassays, such as ELISA.

A range of histamine release assays have been developed, most of which rely on the assessment of mediators released from peripheral blood basophils. These basophils can be incubated with differing allergen and the release of proinflammatory mediators analysed to establish sensitisation to the allergen in question. Assessment of histamine release after basophil-allergen challenge has previously been demonstrated to accurately establish allergens in the horse (Morán et al., 2010a; Wagner et al., 2008). This method has successfully been used in detecting allergen associated with IBH (Langner et al., 2008; Wagner et al., 2008) and sEA (Moran et al., 2012; Morán et al., 2010b, 2010c).

More commonly, serological methods are employed to profile IgE either using polyclonal (Eder et al., 2000; Halliwell et al., 1993b; Kalina et al., 2003) or

monoclonal anti-IgE (Langner et al., 2008; Morgan et al., 2007). The specificity of anti-IgE antibodies is essential, as IgE is present in a much lower concentration compared with other immunoglobulin isotypes, those present in higher levels, such as IgG, bind to allergen extracts in higher concentrations than IgE (Langner et al., 2008; Morgan et al., 2007). To date, accurate and increased specificity is only noted in assays employing monoclonal antibodies. Another limitation for many sIgE assays is the overuse of allergen extracts as opposed to pure proteins, resulting in cross-reactivity between proteins, particularly those of the same genus. Furthermore, some researchers have not normalised protein samples to a set protein concentration, also resulting in questionable results. Ergo, the use of these protein extracts alone with a polyclonal anti-IgE antibody is likely to result in false-positives, inconsistency between assay batches and further complexities to data analysis. Many authors have concluded serological sIgE assays are not reliable and should not be employed in equine allergen diagnostics (Eder et al., 2000b; Morgan et al., 2007). Therefore, the identification of many allergen associated with allergies and inflammatory diseases have been unelucidated to date. More recently, the use of pure and recombinant proteins in combination with MAbs has shown much greater accuracy and diagnostic potential (Marti et al., 2008). This method has since been employed with mathematical modelling for classification, proving to be incredibly precise in both its diagnosis and allergen identification (Marti et al., 2015).

In conclusion, IgE assays must be interpreted with caution while also considering clinical signs and medical history. There are countless reports of

healthy horses testing as sensitised via *in vitro* histamine release assays and during intradermal testing while never showing any signs of disease (Marti et al., 2008). This has led many to believe sensitisation does not necessarily correlate with disease alone. These methods are however of great value in identifying causal allergens in horses presenting with clinical signs of a specific disease. Comparison of research assays as well as those commercially available have demonstrated poor to moderate correlation at best (Lorch et al., 2001; Marti et al., 2008; Morgan et al., 2007). Furthermore, skin prick tests which are considered the gold standard in human assessment have been shown to be inconsistent in the horse, being poorly repeatable, resulting in false positives and being difficult to interpret (Lebis et al., 2002). Due to the aforementioned limitations there is currently no “gold standard” sIgE diagnostic test for the horse, however, the use of pure proteins in conjunction with mathematical modelling shows clear promise for further development and future application.

2.3 Protein microarrays

Protein microarrays are a miniature solid-phase immunoassay the size of a microscope slide on which thousands of separate proteins can be spotted. The interaction between these proteins and specific immunoglobulin isotypes can be tracked using techniques such as fluorescence and algorithms applied to analyse image processing and pattern recognition (Jambari et al., 2017).

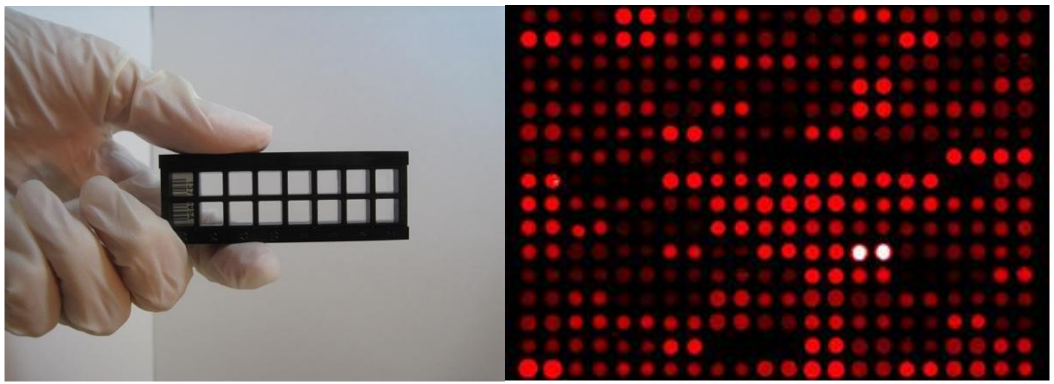

Plate 1: (a)16 pad nitrocellulose slide (b) Fluorescent reading of protein spots

Protein microarrays, particularly those utilising recombinant proteins, have a unique ability to ascertain and monitor specific immunoglobulin profiles to certain allergen in hypersensitised individuals, using a singular test on a miniaturised scale (Harwanegg et al., 2003). The wide-scale processing of this quantity of allergen would be impossible if utilising cumbersome techniques such as ELISA due to the time-consuming nature of this method, the quantity of sample and reagents required, and the economic implications this would have (Fall and Nießner, 2009). The microarray platform circumvents these limitations, while enabling multi-allergen testing to assess complex sensitisation profiles in a single assay (Harwanegg et al., 2003).

It is now widely accepted that the diagnostic accuracy of protein microarrays with selected allergen is comparable to that of standard laboratory tests, such as Unicap, ELISA, CAP/RAST, Immunocapture and immunoblot test, providing a sensitive, reproducible and highly quantitative alternative to these methods (Jahn-Schmid et al., 2003; Jeon et al., 2018; Renault et al., 2011). Protein microarrays have seen wide-scale application in human allergy

diagnostics, but minimal application in the veterinary sector, particularly in the horse.

Since the initial development of protein microarrays in 1999, this method has flourished both in terms of technological development and application of various platforms (MacBeath et al., 1999). This has had an immense impact on life sciences, enabling researchers to elucidate the complex biological systems surrounding specific diseases and immunochemical pathways. All protein microarrays require several essential operational steps to ensure appropriate performance. The primary factors are protein library construction and microarray fabrication. In the last decade alone, microarrays have changed from a basic analytical tool for application in profiling proteins for biomarkers, to informing diagnostics, drug discovery and vaccine creation. Crucially, each application takes full advantage of the microarray's key features: miniaturised platform and parallelisation. The application of this method in human allergology has been extensive, seeing IgE profiling for food allergies (peanut/milk/eggs), latex, hay fever, rhinitis and asthma (Cabral, 2010; D'Urbano et al., 2010; Ebo et al., 2010; Lin et al., 2012; Patelis et al., 2012; Sanz et al., 2011; Shaoqing et al., 2011); however its implementation in equine allergology has been limited to date (Marti et al., 2015).

2.4 Equine application of protein microarrays

Marti et al., (2015) utilised pure proteins and extracts relevant for the assessment of Insect Bite Hypersensitivity in combination with mathematical modelling for disease classification. The authors confirmed the high discriminatory power of this approach to IBH diagnostics and emphasised the benefits of this technique in identifying specific sensitisation profiles in at risk individuals, which may be utilised for clinical diagnosis. More recently, Einhorn et al. (2018) attempted to refine the protein microarray method to explore sensitisation profiles in the horse, as many eliciting molecules are currently unknown. The authors concluded that antigen microarrays provide novel information on the allergen sensitisation patterns. Both studies utilised the monoclonal mouse anti-horse IgE antibody clone 3H10 with Marti et al. (2015) using the original described mAb, and Einhorn et al. (2018) using the commercially available Bio-Rad clone. These two studies emphasise the enormous benefits this technique offers in assessing allergies and inflammatory diseases in the horse, elucidating aetiology, pathogenesis, as well as, revealing sensitisation patterns for diagnosis and the development of treatments. With the increased ability to purchase and produce recombinant proteins, this technique is the ideal option for component resolved diagnostic application.

As discussed, the ever-developing applications of protein microarrays have enabled the accurate identification of novel biomarkers in several diseases. Although the previous limitations associated with the identification of biomarkers in sEA have been well discussed, the increased sensitivity of microarrays in Ig detection and novel application of classification methods

pave the way for further exploration. The data derived from sEA sIgE biomarker studies has been contradictory to date due to the limited panel of allergen utilised, and primarily focus on extracts as opposed to pure/recombinant proteins and implementing cut off limits. In this thesis, I utilise these developing technologies and apply them to develop a microarray platform to profile sIgE and identify sensitisation profiles in sEA affected-horses, as well as identify allergens in environmental samples via the competition method.

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3.0 Aims of the study

To develop a comprehensive antigen microarray comprising of common equine environmental proteins to identify allergens associated with sEA, explore its diagnostic potential and evaluate the influence of hay preparation techniques on IgE-protein binding. This could provide superior diagnosis, improve allergen-avoidance regimes, and enable diagnostic and therapeutic advancement. Building on the latest advancements in microarray technology and applying this to sEA, the specific objectives were:

Develop and validate an antigen microarray for IgE profiling of BALF and sera in sEA-affected and healthy horses, ensuring the platform is optimised for printing, incubation, immunolabeling, biological fluid source, concentration techniques, reproducibility, and specificity. [*paper I*]

Utilise the developed microarray for wide scale allergen profiling in sEA-affected, multiple-hypersensitivity and healthy horses, developing mathematical modelling procedures for disease classification and the identification of sEA-associated allergen. [*paper II*]

Develop a microarray competition technique to identify specific allergen sources in hay samples, and the effect of pre-treatment on IgE-protein binding. [*paper III*]

4.0 Paper I

Hypothesis: Antigen microarrays can be optimised to enable large-scale IgE mapping and identification of allergens associated with sEA.

DOI: 10.1111/jvim.15564

STANDARD ARTICLE

Development of a comprehensive protein microarray for immunoglobulin E profiling in horses with severe asthma

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Keywords: *severe equine asthma; horse; protein microarray; IgE; allergen.*

Abstract:

Background - Severe equine asthma (sEA) is a prevalent, performance-limiting disease associated with increased allergen-specific immunoglobulin E (IgE) against a range of environmental aeroallergen.

Objective – To develop a protein microarray platform to profile IgE against a range of proven and novel environmental proteins in sEA-affected horses.

Animals - Six sEA-affected and six clinically healthy warmblood performance horses.

Methods - A protein microarray (n=384) was developed using protein extracts and purified proteins from a large number of families including pollen, bacteria, fungi and arthropods associated with the equine environment. Conditions were optimized and assessed for printing, incubation, immunolabeling, biological fluid source, concentration techniques, reproducibility and specificity.

Results – This method identified a number of novel allergens, while also revealing an association between sEA and pollen sensitization. Immunolabeling methods confirmed the accuracy of a commercially available mouse anti-horse IgE 3H10 source (R^2=.91). Biological fluid source evaluation demonstrated both serum was as effective as BALF in establishing a specific IgE profile (average R^2=.75). Amicon centrifugal filters were found to be the most efficient technique for concentrating of BALF for IgE analysis at 40-fold. Overnight incubation increased sensitivity, while maintaining the same sensitization profile as a 3-hour incubation. Reproducibility was demonstrated (R^2=.97), as was specificity via protein inhibition assays. As expected, arthropods, fungi and pollens have shown the greatest discrimination between sEA-affected and non-affected horses.

Conclusion - Here we have established that protein microarray can be used for large scale IgE mapping of allergens associated with the equine environment. This technology provides a sound platform for specific diagnosis, management and treatment of sEA.

1.0 Introduction

Severe equine asthma (sEA) is a performance limiting, allergic response to inhaled allergen in genetically predisposed horses, that effects approximately 14% of the UK equine population [1–3]. Allergen exposure in affected horses results in small airway inflammation, mucus hypersecretion and bronchoconstriction; altering pulmonary resistance, dynamic compliance and pleural pressure [4–6]. The predominant source of these aeroallergens is the organic dust portion of forage and bedding, which contains fungi, bacteria, pollen and arthropods [7–11]. Removal from the aeroallergen-rich stabling environment results in a level of remission [12], but owner compliance is limited due to seasonality, competition schedule, health issues and nutritional demands. Treatment with corticosteroids and bronchodilators provide short-term relief; however, such therapeutic approaches have been associated with undesirable adverse effects and their use is prohibited under *Fédération Équestre Internationale* and jockey-club rules [13,14]. Allergen avoidance is the cornerstone to prevention and effective treatment, however the efficacy of the latter approach relies on identification of causal allergens[15]. Currently, the major obstacles to diagnostic and therapeutic developments include a) major limitations in the number of allergens screened to establish causal agents, and b) the lack of a clinically applicable *in vitro* test.

The pathogenesis of this condition remains unclear; however, several studies have demonstrated the involvement IgE immunoglobulin E (IgE) through *in vitro* histamine release assays [16–18], natural hay and straw challenges [19],

intradermal testing [20] and specific IgE (sIgE) analysis of bronchoalveolar lavage fluid (BALF) and sera [21,22]. sIgE assays suggest that *Aspergillus fumigatus, Alternaria alternate, rAsp f 8*, *Tyrophagus putrescentiae*, *Saccharopolyspora rectivirgula,* Asp f 1/a, *Aspergillus terreus, Eurotium amstelodami, Geotrichum candidum* and *Wallemia sebi* are implicated in the etiology of sEA [8,21,23–25]. Although a vast number of recombinant proteins are available [26], advancements in causal allergen identification has been limited due to the viability of testing with classic methods, such as ELISA, which are time-consuming, expensive, and require large quantities of samples and reagents [27].

In recent years, protein microarrays have been gaining popularity in allergy diagnostics due to their ability to assess the interaction of thousands of proteins with specific immunoglobulin isotypes using techniques such as fluorescence, on a miniaturized scale; a technique known as microarray profiling [28]. This circumvents the aforementioned limitations associated with techniques, such as ELISA, enabling multi-allergen testing to assess complex sensitization profiles. Furthermore, with specific allergen these tests show similar sensitivity to standard laboratory methods, including ELISA, UniCAP, CAP/RAST, ImmunoCAP and immunoblot test [29–31]. Previously published sensitivity and specificity values using protein microarrays have demonstrated the high discriminatory power of the protein extracts and pure recombinant *Culicoides* proteins associated with insect bite hypersensitivity in the horse [22].

The aim of the presented study was to develop, and demonstrate, that widespread allergen profiling using microarray methods enables fast and accurate IgE profiling of sEA. Furthermore, we wanted to analyze the correlation between BALF and serum specific IgE profiles, a crucial consideration with respect to diagnostic sample requirements. Profiling data allows for diagnostic and therapeutic advancements.

2.0 Materials and methods

2.1 BALF and serum samples

Clinical assessment including physical examination, pulmonary function tests and bronchoalveolar lavage fluid (BALF) cytology was used to define the inclusion and exclusion criteria for selection of six horses with sEA and six control horses [5]. BALF was collected as previously described [32], filtered through a 100 ml syringe filter (Biocomma, Shenzhen, China), and decanted into 10 ml aliquots in 15 ml centrifuge tubes with the addition of Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free (product # 13437766). The mixture was gently agitated and incubated at 4°C for 10 min prior to the addition of 2.5 ml glycerol (Fisher Scientific, Leicestershire, UK) and stored at -80°C until analysis. To concentrate, BALF was thawed, maintained at 4°C and filtered with a Sartorius Stedium 0.45 µm filter syringe (product # 17598). BALF samples were then concentrated in an Amicon Ultra-15 centrifugal filter (product # UFC905024) and used immediately. Blood was collected and serum prepared as previously described [33], prior to storage at -80°C until analysis, at which time the samples were thawed at room temperature and placed on ice.

Bronchoalveolar lavage was concentrated via one of two popular techniques. Firstly, through lyophilization, after being passed through a PD-10 desalting columns (GE Healthcare, 17-0851-01) following the manufacturers protocol, thus ensuring the removal of excel salts and glycerol (Pringle et al., 1998; Wilkie and Markham 1979). Secondly, through centrifugal filtration using an Amicon Ultra-15 (previously known as Centricon-10) following the manufacturers protocol (Peebles et al., 2001; Koh et al., 2001). Both methods allowed BALF to be concentrated to the required quantity.

2.2 Proteins, printing and hybridization

In order to maximize utility, the microarray was designed to be as comprehensive as possible by containing extracts and pure proteins from a wide range of protein families derived from predominantly, fungi, bacteria, pollen and arthropods. The extracts and pure proteins were obtained from commercial suppliers, produced in-house and from donations. Due to the limited commercial availability of some bacterial and fungal protein extracts, it was necessary to produce them in-house. Lyophilized purified samples of the desired strain were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (https://www.dsmz.de) and grown in 150 ml of liquid media according to the supplier's recommendation (250 ml Erlenmeyer flask). Once grown, the media were centrifuged in 50 ml tubes at 4000 × g for 10 min, supernatant was carefully removed before washing the individual pellets in 5 ml of PBS. The tubes were centrifuged at 4000 × g for 10 min, supernatant removed and 1 ml of lysis buffer solution added to each

tube (PBS, 0.5% TritonX-100 w/v and Thermo Scientific EDTA-free protease inhibitor mini-tablet). The re-suspended pellets were pooled into a single 50 ml centrifuge tube, placed on ice, and sonicated using an MSE Soniprep 150 (15 seconds sonication with 30-second cooling periods in-between for 10 cycles). Subsequently, the solution was filtered through a Nalgene 0.45 um syringe filter (product # 190-25-45) and protein content quantified using a Pierce BCA Protein Assay Kit (product # 23225). The remaining solution underwent lyophilization, was re-suspend in MiliQ water with 10% glycerol (filtered through a 0.02 um syringe filter) and normalized to 1mg/ml protein and stored at -80 ^{o}C.

Bronchoalveolar lavage concentration work was initially conducted using slides described in Marti et al., 2015 consisting of extracts (n=240) and pure proteins (n=120) from a range of protein families including amongst others fungi, pollen and arthropod to establish the optimal BALF concentration to be utilize in subsequent development work. In-house extracts not present in the initial array setup were initially trialled by printing normalized samples (1 mg/ml protein) onto 16 pad FAST™ slides (Whatman Schleicher & Schuell, Dassel, Germany) using a QArraylite arrayer (Genetix, UK). After sample selection, a new set of 384 proteins (supplementary data) were printed in a professional setting using a Marathon microarrayer (ArrayJet, Roslin, Scotland) printer essentially as described in Marti et al., (2015) with an approximate spot size of 200 μm diameter and replicated with even spacing two times across each of the individual 16 pads into two identical blocks to final spot density of 12,288 spots/slide. For alignment and quality control, spots of Cy3, Cy5 and PBS were

printed onto each slide. Once printed, slides were blocked for 3 h at 37 °C in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA), washed three times for 2 min in PBS containing 0.05% (w/v) Tween-20, followed by five times 1 min washes with MiliQ water, and dried by centrifugation (MSE Mistral 3000i, Sanyo, UK) at 1000 × g for 10 min at room temperature.

Slides were fitted with Proplate slide modules (Grace Bio-Labs, product # 204862) and washed three times (60 second dwell time) with PBST (0.2 %). Samples (BALF / serum) were diluted 1:2 with 4 % BSA in PBST (Tween at 0.4% w/v) containing Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free (product # 13437766)(1 tablet in 5 ml), which has previously been passed through a Whatman 13 mm, 0.45 µm filter syringe (product # 6784-1304). 100 µl of prepared sample was added to each well, excluding well 4, which was used as control and filled with 100 µl of the dilution solution (1:2) in PBS. The Proplate was fitted with an adhesive seal strip and incubated for 16 h at 4°C on the Stuart mini see-saw rocker (SSM4) at 13 oscillations / minute. Slides were washed three times with PBST (Tween at 0.05%) using the BioTek plate washer and incubated for 2 h at 37°C in a ThermoHybaid (HyPro 20) at AVS 3 with 100 µl per well of mouse anti-horse IgE (BioRad, #MCA5982GA) 1:400 in 1% BSA in PBST (Tween at 0.2% w/v), washed a further 3 times with PBST (Tween at 0.05%) and incubated for 1h at 37°C in the ThermoHybaid with 100 µl per well of DyLight 649 conjugated anti-mouse IgG1 (Rockland, Product #610-443-040) 1:400 in 1% BSA in PBST (Tween at 0.2% w/v). The slide was then washed 3 times in PBST (Tween at 0.05%) followed by three

washes with Milli-Q water, and dried via centrifugation at 300 × g for 10 mins (Mistral 3000i, rotor 43124-708).

2.3 Protein Inhibition Assay

In order to test the IgE specificity of IgE-protein binding, a protein inhibition assay was performed to assess cross-reactivity. Proteins, singularly or in a mixture, exhibit similar inhibition effects on IgE binding to the same or neighboring proteins. *Ergo,* creating protein inhibition groups containing several proteins found to bind to the patients IgE, can be used to assess these affects. Two different protein inhibition groups were used, each containing two different proteins. Group 1 consisted of *Blattella germanica* (Bla g 1) and *Rumex crispus* (Rum cr), and group 2 consisted of *Penicillium notatum* (Pen ch) and *Acinetobacter gerneri* (Aci g). Pooled serum was spiked with the aforementioned proteins in serial dilution and its effect on related and neighboring proteins evaluated. A protein inhibition assay enables the confirmation of specificity of an antibody against the target protein, and is therefore conducted with several proteins to confirm both the antibodies specificity to the target protein and assess potential cross-reactivity [34].

2.9 Data Analysis

Processed slides were scanned in a Genepix 4000B (Molecular devices, USA) with the *photomultiplier* tube (PMT) settings at 440 and 310 at 635 and 532 nm respectively and saved as TIF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. Digital fluorescence units (DFUs) were calculated for each spot by

subtracting local background from the median fluorescence value of the spot. One pad per slide contained all reagents with addition of PBS instead of serum for control purposes, these results were deducted from samples on the same slide to account for any protein autofluorescence and non-specific binding. Further analysis and data presentation were carried out using Microsoft Excel. Average florescent unit readings for each protein were compared between sEA and control groups using a conventional Z-test in Excel (Microsoft, USA). The Benjamini-Hochberg method was used to account for false discovery at a rate of .05. Benjamini-Hochberg corrected values were considered significant at $P < .05$. Linear regression (coefficient of determination) of IgE DFUs for all proteins (n=384) was used to establish the relationship between BALF Vs serum, reproducibility of results and varying mouse anti-horse IgE sources. Bronchoalveolar lavage fluid concentration techniques were compared using ANOVA.

3.0 Results

3.1 Optimizing serum incubation conditions

3.1.1 BALF concentration techniques

BALF concentration employing Amicon and PD10/lyophilizing methods were compared using a BALF pool from six horses (n = 3 sEA; n = 3 control). Total IgE for each protein group was used to compare concentration methods (figure 1A), revealing no significant difference ($p<0.05$) between concentration techniques. The Amicon concentration method was used to evaluate optimal BALF concentration via total IgE fluorescence for each protein group, revealing that a plateau was reached at 40-fold concentration (figure 1B).

Therefore, all subsequent BALF concentrations were carried out via Amicon filtration to a 40-fold final concentration.

3.1.2 Incubation time

Two conditions were tested for optimal serum incubation times using a serum pool from six horses (n = 3 sEA; n = 3 control); 3 h at 37°C as previously used for equine serum [22] and overnight (16 h) at 4°C which has previously been shown to be more sensitive in human studies [34]. As shown in figure 2, the IgE profile between the two incubation times significantly correlated ($R^2 = 0.70$), however, when the serum was incubated for 16 h at 4°C, it was more sensitive with 28.08% of proteins showing positive reactions, compared to the 4 h incubation, which showed 16.44% of proteins with positive reactions (data not shown). *Ergo*, subsequent serological incubations were conducted overnight at 4°C to increase sensitivity.

3.1.3 Comparison of specific IgE in BALF and serum

Bronchoalveolar lavage samples concentrated by Amicon (40-fold) were compared with serum from six horses (n = 3 sEA; n = 3 control) and correlation coefficients calculated for each separate protein group. Strong correlations were demonstrated between BALF and serum (table 1), ergo, all subsequent incubations were conducted with serum as it is far easier to obtain, is less invasive, more economical and stable to transport. It should be noted that the strength of correlation seemed to be partly dependant on the protein group (fungal vs bacterial vs pollen vs arthropod). Horse 5, a clinically healthy horse, showed poor BALF x serum correlations across all protein groups, which was

thought to be a result of the horse recently changing to a different barn on the same yard, and therefore localized IgE production in the lung due to environmental allergen correlated poorly with serological IgE [23,35].

3.2 Reproducibility

3.2.1 Printing lot variation

The effect of printing lot on reproducibility of the protein microarray was assessed using two microarray slides printed on the same day. Sera from three sEA and three control horses was hybridized on the two slides simultaneously. Fluorescence values from replicate arrays were evaluated using linear regression and showed R^2=0.97, demonstrating the fluorescence values from the array were repeatable between printing lots (data not shown).

3.2.2 Comparison between monoclonal anti-horse 3H10 sources

Two mouse anti-horse IgE monoclonal antibodies (derived from the 3H10 clone) were compared via linear regression of the fluorescence values using a serum pool from six horses (n = 3 sEA; n = 3 control), this included the original 3H10 from Wilson et al., (2006)[36] and the commercially available BioRad 3H10 (#MCA5982GA).

As shown in figure 3, the fluorescence values from the array showed a correlation coefficient of R^2=0.91, demonstrating a significantly similar IgE profile.

3.3 Specificity – peptide inhibition assay

Interestingly, reduced fluorescence from proteins other than those targeted was also observed, indicating either non-specific inhibition or some similarity between the allergenic components of the proteins. Group 1 inhibition group showed no non-specific binding in the bacteria, arthropod or fungi groups, however, cross-reactivity was seen among grass pollens – most notably *Cynodon dactylon*, *Rumex crispus*, *Zea mays* and *Anthoxanthum odoratum*. Group 2 inhibition group demonstrated no non-specific binding in the bacteria, arthropod and pollen groups, however, cross-reactivity was seen between *Aspergillus niger, A. versicolor, Penicillium expansum, P. notatum, A. nidulans* and *A. fumigatus* (figure 4).

3.4 Allergen comparison

Whether the prototype array was able to identify novel allergens associated with sEA was assessed using Z-tests between six sEA and six control sera samples. The results shown in table 2 confirmed the ability to conduct IgE profiling and identify potential sEA allergens via microarray methods. As expected, it confirmed the presence of fungi and mite as main reactants in the sEA population, while also identifying an association with pollen which has not previously been implicated.

4.0 Discussion

We have previously demonstrated the sensitivity and specificity of microarrays in the diagnosis of insect bite hypersensitivity [22], confirming the high discriminatory power of complex extracts and pure recombinant *Culicoides*

proteins associated with the allergy. Based on these principles, an array was constructed to enable multi-allergen testing and assess the complex sensitization of profiles associated with sEA in a single assay, based on equine environmental proteins. The use of protein extracts was essential as so few proteins have been assessed in relation to sEA to date; therefore, in the early stages of development it was wise to use a range of extracts to maximize coverage, in combination with pure proteins, where available or where the allergen has been previously associated, thus simultaneously maintaining specificity. Furthermore, we observed comparable accuracy between natural extracts and recombinant allergens, whereas some authors suggested the use of the recombinant component alone may be insufficient for some allergens [37]. Although the eventual goal will be to move towards component resolved diagnostics (CRD) utilizing individual allergen molecules for increased sensitivity and minimizing cross-reactivity, the genus/species must be identified to enable the production of pure proteins. CRD offers vastly increased accuracy over routine diagnostic tests (skin prick and specific IgE determination) [38], and enables the accurate selection of allergens to be used for allergen immunotherapy [39]. Moreover, the identification of sensitization to pure proteins will assist in diagnostic and therapeutic advancement. This microarray approach has several advantages: allows substantial allergen profiling with minimal sample, collection of less invasive readily obtained *in vivo* samples, permits automation and enables the generation of mathematical predictive models to assist in clinical allergy diagnosis.

Protein microarray methods primarily consist of two steps: firstly, the printing of proteins onto the nitrocellulose slides, and secondly the profiling of equine IgE. Printing methods are well established; therefore, the latter was optimized to enable analysis of sEA BALF and serum samples. Although it has been suggested that developing technology means sensitivity is such that many immunoglobulin isotypes in BALF can be assessed un-concentrated to the nanogram or microgram, it is often not possible to detect allergen-specific IgE in BALF due to the low concentration of this isotype present [23,40]. Therefore, concentration techniques must often be employed to assess BALF IgE. Lyophilization [41] and centrifugal filtration methods [42,43] have been successfully utilized to concentrate BALF for immunoglobulin analysis, however certain techniques such as ammonium sulphate precipitation, can result in denaturation of the liable epitope(s) [44]. Similarly, lyophilizing BALF samples without desalting results in high levels of sodium chloride, which can denature proteins. The most commonly utilized BALF concentration technique is the centrifugal filter (Amicon Ultra-15 or Centricon-10), to a 10-fold concertation of immunoglobulin analysis [42,45–49], however, even this technique results in a 20% loss of specific IgE and IgG [46]. On collection of BALF for immunoglobulin analysis protease inhibitors must be added to prevent proteolytic cleavage of proteins, which would otherwise leave the immunoglobulin unviable. Similarly, immunoglobulin in BALF is greatly affected by freeze-thaw cycles, so the inclusion of a cryopreservative in the form of glycerol has been shown to be effective [50]. Therefore, glycerol was added as a cryoprotectant to a final concentration of 20% to help stabilize the proteins and prevent formation of ice crystals during freezing that destroy protein structure. Unfortunately,

samples containing cryopreservative tend to thaw during lyophilization, meaning it was not possible to achieve a 40-fold concentration using this technique. To avoid this and remove sodium chloride concentration, samples underwent a buffer exchange using a PD-10 column prior to lyophilization. Bronchoalveolar lavage concentration levels and techniques were trialed, varying hybridization temperatures and times were assessed, and BALF V's serum analyzed. The greatest binding capacity was observed with overnight incubation at 4°C. When using BALF the greatest binding was seen using the Amicon filter and the PD-10 elution column/lyophilizing at a 40-fold final concentration. The Amicon filter was quicker, easier, reduced risk of contamination and had previously been utilized, therefore this technique was selected. Interestingly, during optimization a biased response was observed toward pollens. Plants are polyploid and show a great number of gene duplications, hence high cross reactivity between species are generally observed. It is our experience that pollen response in humans and other animals is always amplified. Due to the significant correlation of BALF and serum (average R^2=0.75), serum was used in subsequent analysis due to ease of use. Previous work comparing the specific IgE profile of BALF and serum have utilized ELISA techniques, has been limited, and, to date, the results have been contradictorily. The authors concluded that although BALF may be valuable for analysis, serum was of little clinical relevance [21,23]. Here we demonstrated the ability to profile unconcentrated serum instead of BALF to assess potential allergens. This has several advantages as serum is far easier to collect, store and prepare for analysis in comparison to BALF. Collection is also less stressful for the horse. Moreover, it holds further

potential in the use of diagnostic microarrays as serum is far more stable to ship for analysis [51].

Repeatability is an essential factor in the development of new diagnostic tests. *Ergo*, the effect of printing lot and Mouse anti-horse IgE 3H10 sources were assessed. These results confirmed the reproducibility between printing lots (average R^2=0.97). The original Mouse anti-horse IgE 3H10 used was that from the Wilson et al., (2006) study. The commercial availability of antibodies is essential in diagnostic tests, therefore the Wilson et al., (2006) 3H10 clone was compared with the commercially available BioRad 3H10 (#MCA5982GA), confirming reproducibility with the commercially available clone (R^2=0.90).

Specificity is an important aspect of protein microarrays, which was confirmed by a protein inhibition assay. In this assay some cross-reactivity was seen, predominantly with grass pollens, as well as, *Aspergillus* and *Penicillium*. Sridhara et al., (1995) reported pollens from grasses (Poaceae) often show high immunological cross reactivity, potentially indicating common antigenic / allergenic component(s) [52]. Furthermore, cross reactivity was identified between the genus *Penicillium* and *Aspergillus*, which is expected as taxonomically, the genus *Penicillium* and *Aspergillus* have many similarities, as both produce and contain galactomannans with similar galactofuranosyl and immunogenic side-chains. It is worth noting cross-reactivity in the fungi group was only seen with whole protein extracts, emphasizing the importance of including pure proteins. Analysis of human sera in a variety of assays has indicated that *Aspergillus fumigatus* contains determinants in common with

Cladosporium, *Candida, Alternaria*, *Trichophyton* and *Epidermophyton* [53], however cross-reactivity was not identified on this occasion.

Several allergens of interest identified here were consistent with those previously identified as sEA-associated through ELISA, western blot and RAST methods (*Aspergillus fumigatus, Alternaria alternate, Eurotium amstelodami,* and *Geotrichum candidum*). This was the largest panel of proteins tested with a controlled sEA group to date, and thus revealed new and relevant allergens. Several sEA-associated allergens identified in this study have previously been associated with allergic asthma in the human (*Dermatophagoides farinae; Blattella germanica; Aspergillus restrictus; Dermatophagoides pteronyssinus*). The novel sEA-associated allergen identified in this study strongly implicate fungi and mite as the main reactants, as well as, revealing a previously unidentified reaction with pollens. This confirms the future potential of specific IgE as a biomarker for the serological diagnosis of sEA.

The results of this study have clearly established a reliable protein microarray for large scale IgE profiling of equine environmental proteins, confirming identified sEA-associated allergens and elucidating a range of previously unidentified allergens. The technique is sufficiently sensitive and specific to differentiate between sensitized allergens in sEA and control horses. Furthermore, the developed serological assay enables accurate identification of an individual horse's sensitization profile. This information provides a reliable, fast and repeatable method for screening a wide variety of potential

allergens found in the stable environment in a miniaturized and affordable format, while offering a platform to support management and treatment of this debilitating respiratory disorder in horses.

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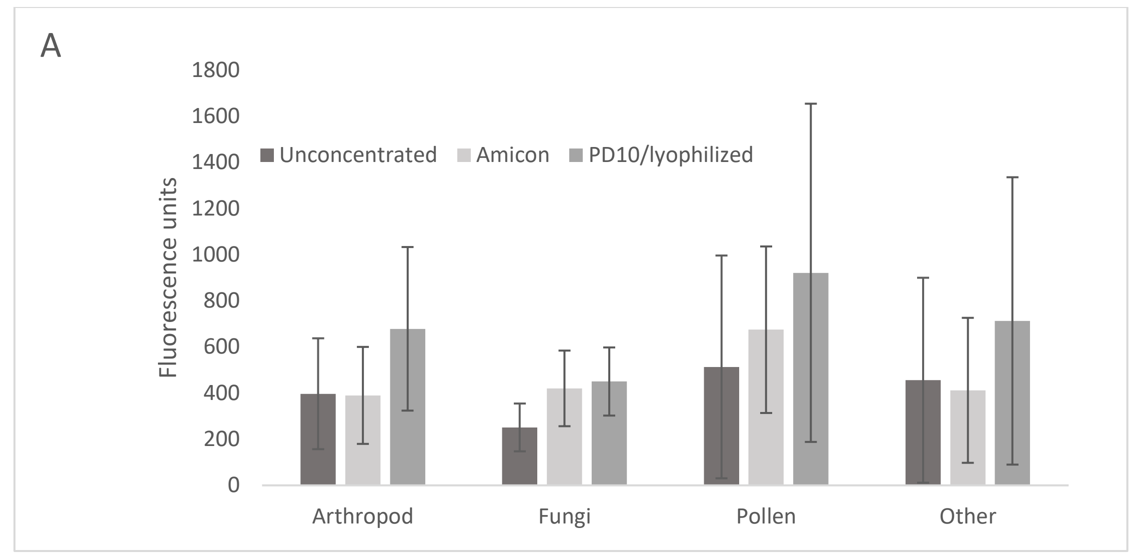

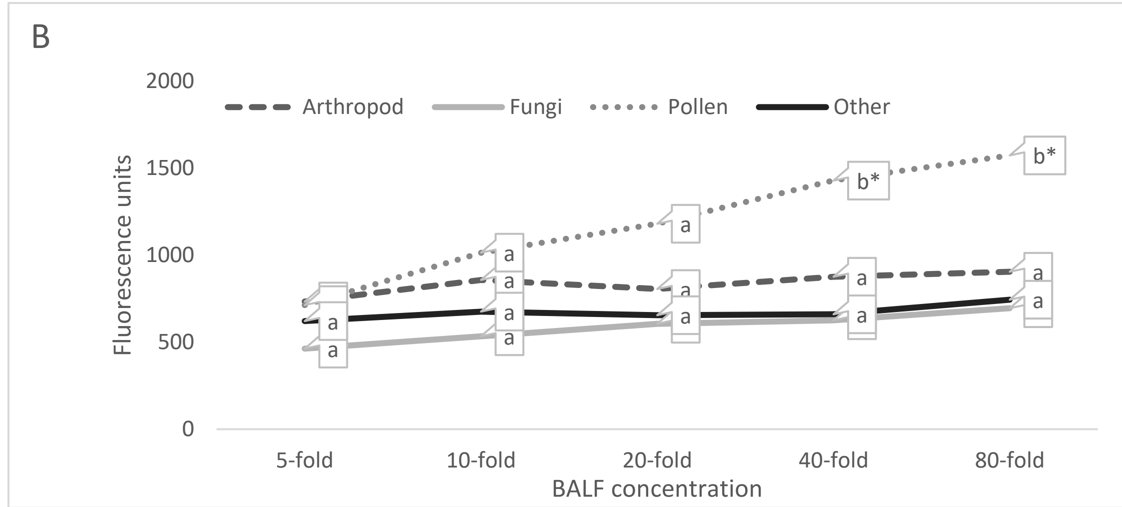

Figure 1A: BALF concentration optimization. Average BALF IgE fluorescence units for each protein group demonstrating concentration efficacy for unconcentrated, and concentrated (10-fold) using either Amicon filter or PD10 columns/lyophilizing, A one-way ANOVA for each protein group using a BALF pool from six horses (n = 3 sEA; n = 3 control) showed there was no significant difference ($p<0.05$). **Figure 1B:** Average BALF IgE fluorescence units for the main protein group at various concentrations. Concentration via Amicon filtration using a BALF pool from six horses (n = 3 sEA and 3 control). Groupings included arthropod, fungi, pollen and other; which largely consisted of food and environmental proteins. Significant differences of each protein

group were calculated individually by one-way ANOVA with Tukey's HSD (* = $p<0.05$). *Means* that have no *superscript* in common are *significantly different* from each *other.*

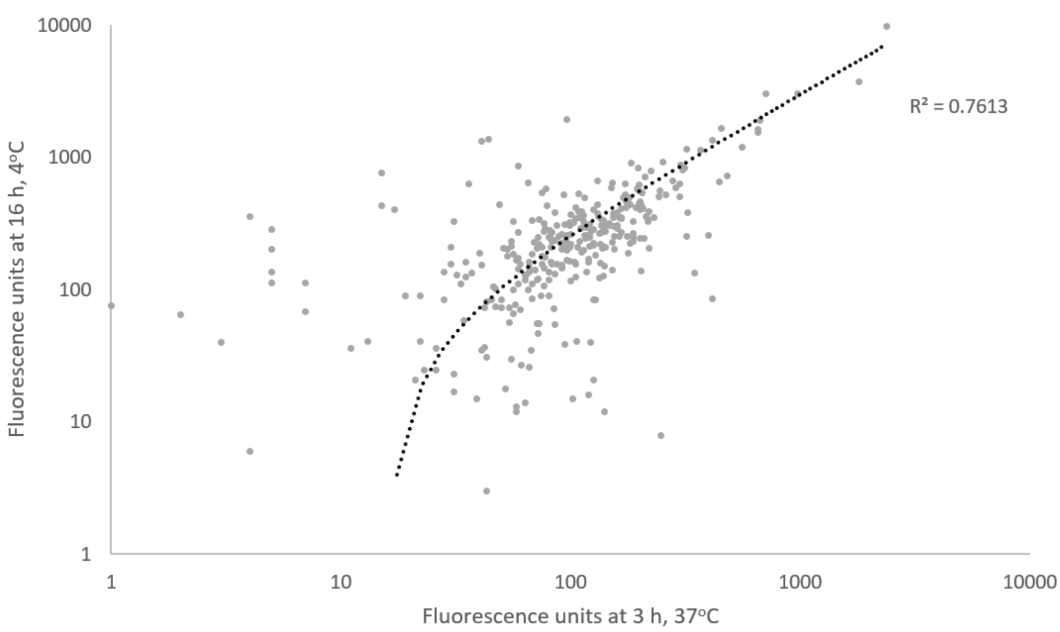

Figure 2: Comparison of incubation methods. Linear regression of microarray IgE fluorescence profiles (n=384 proteins) of pooled sera (n = 3 sEA and 3 control) incubated for 3 h, 37°C and 16 h, 4°C on a log scale.

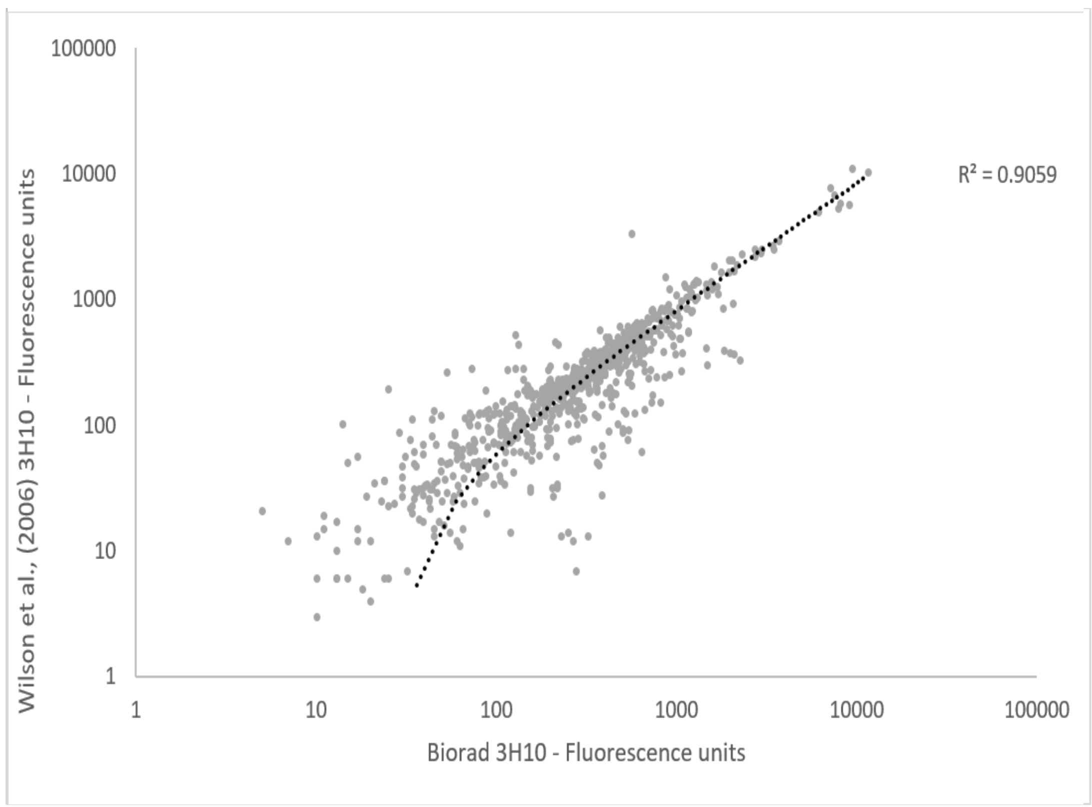

Figure 3: Comparison of secondary antibodies. Linear regression comparing protein microarray (n=384) IgE fluorescence unit readings on log scale from pooled sera (n = 3 sEA; n = 3 control) using two mouse anti-horse IgE 3H10 clones; Wilson et al., 2006 and BioRad (product # MCA5982GA).

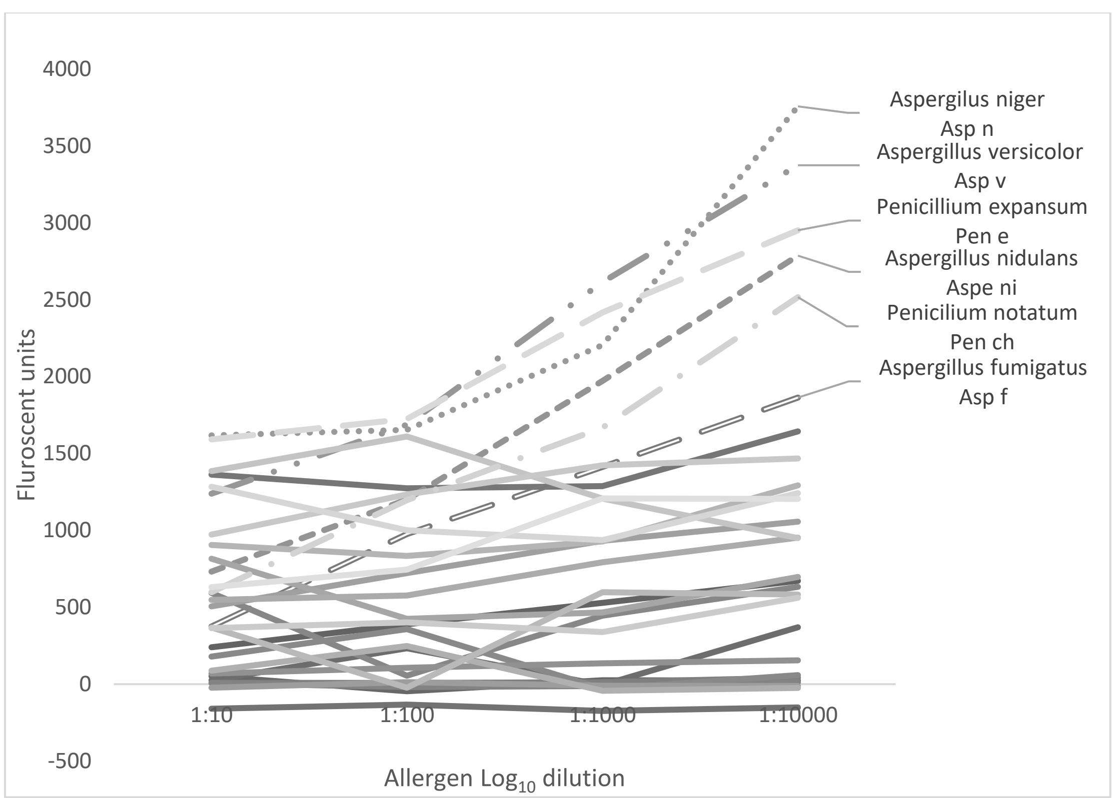

Figure 4: Group 2 inhibition group. Post incubation with the target protein (*Penicillium notatum* Pen ch) at varying concentrations (e.g. 1:10), demonstrates that there is altered IgE fluorescence readings for various proteins simultaneously with the target protein between serial dilutions. Therefore, *Penicillium notatum* cross reacts or non-specifically binds with *Aspergillus niger, A. versicolor, Penicillium expansum, P. notatum, A. nidulans* and *A. fumigatus,* indicating cross-reactivity/non-specific binding.

Table 1: Regression coefficients between total specific IgE fluorescence values between protein groups in sera and BALF for each of the 3 sEA-affected horses (horses 1-3) and 3 non-affected (horses 4-6).

	Pollen	**Arthropod**	**Fungus**	**Bacteria**
Horse 1	0.63	0.82	0.71	0.74
Horse 2	0.79	0.96	0.73	0.91
Horse 3	0.65	0.92	0.54	0.88
Horse 4	0.91	0.99	0.56	0.72
Horse 5	0.44	0.58	0.33	0.89
Horse 6	0.87	0.85	0.59	0.98

Table 2: Z-test results with Benjamini-Hochberg corrected p-values showing all statistically significant allergen between the sEA (n=6) and control group (n=6)(p = 0.05).

Name		Benjamini-Hochberg P-value
Dermatophagoides farinae	Der f 2	0.00040
Blattella germanica	Bla g 5	0.00042
Aspergillus restrictus	Asp r 1	0.0016
Linum usitatissimum	Lin us [pollen]	0.0086
Dermatophagoides pteronyssinus	Der p 7	0.011
Blattella germanica	Bla g 5	0.011
Hevea brasiliensis	Hev b 11	0.013
Triticum polonicum	Tri tp	0.015
Hevea brasiliensis	Hev b 5.0101	0.015
Penicillium notatum	Pen ch	0.025
Actinidia chinensis	Act c 5	0.025
Malassezia pachydermatis	Mala p	0.031
Actinidia deliciosa	Act d 11	0.036
Olea europaea	Ole e 2	0.036
Anthoxanthum odoratum	Ant o [pollen]	0.041
Hevea brasiliensis	Hev b 6.02	0.043

Parietaria judaica	Par j 1	0.043
Alternaria alternata	Alt a 1	0.043
Triticum turgidum ssp. durum	Tri td	0.047
Aspergillus fumigatus	Asp f	0.048

5.0 Paper II

Hypothesis: Serological antigen microarrays, in combination with mathematical modelling, can be used for the classification of sEA and identification of associated allergen.

OPEN

Antigen array for serological diagnosis and novel allergen identification in severe equine asthma

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Abstract

Severe equine asthma (sEA), which closely resembles human asthma, is a debilitating and performance-limiting allergic respiratory disorder which affects 14% of horses in the Northern Hemisphere and can be associated with increased allergen-specific immunoglobulin E (IgE) against a range of environmental proteins. A comprehensive microarray platform was developed to enable the simultaneous detection of allergen-specific equine IgE in serum against a wide range of putative allergenic proteins. The microarray revealed a plethora of novel pollen, bacteria, mould and arthropod proteins significant in the aetiology of sEA. Moreover, the analyses revealed an association between sEA-affected horses and IgE antibodies specific for proteins derived from latex, which has traditionally been ubiquitous to the horse's environment in the form of riding surfaces and race tracks. Further work is required to establish the involvement of latex proteins in sEA as a potential risk factor. This work demonstrates a novel and rapid approach to sEA diagnosis, providing a platform for tailored management and the development of allergen-specific immunotherapy.

Keywords: *allergen; horse; mathematical modelling; protein microarray; severe equine asthma.*

Introduction

Severe equine asthma (sEA) is a performance limiting, debilitating condition which is prevalent in 14% of horses in the Northern Hemisphere[1]. The pathogenesis of this condition remains controversial with many contradictory

reports[2,3]; but several studies have indicated the role of immunoglobulin E (IgE) through *in vitro* histamine release assays[4–6], and allergen-specific IgE (sIgE) analyses of bronchoalveolar lavage fluid (BALF) and sera[5, 8,10]. Specific IgE assays suggest that *Aspergillus fumigatus* (*Asp f (extract), rAsp f 8,* Asp f 1/a)*, Alternaria alternate*, *Tyrophagus putrescentiae*, *Saccharopolyspora rectivirgula, Aspergillus terreus, Eurotium amstelodami, Geotrichum candidum* and *Wallemia sebi* may be implicated in the aetiology of sEA[2,3,9–12]. More recently, White *et al.,* (2017) identified 40 potential allergens of interest, from several genera, including fungi, bacteria, pollen and arthropod[13].

sEA diagnosis is presently conducted on clinical history and readily identified clinical signs[14], which have been shown to correlate with sEA severity[15], with ancillary diagnostic tests such as BALF cytology, lung function testing, haematology, and immunological testing used to improve diagnostic accuracy[16]. While several studies have addressed the potential benefits derived from *in vitro* allergen assessment in diagnosis of sEA (Tahon et al., 2019; Tilley et al., 2012; White et al., 2019) , commercial application has been hampered due to a lack of statistical approaches for clear disease classification, and the limited range of allergens tested to date.

More recently, White *et al.,* (2019) developed microarray methods to enable IgE profiling in sEA-affected horses, elucidating previously unidentified causal allergens and demonstrating a strong correlation between BALF and sera specific IgE profiles, which was partially dependent on the broad range of allergen source (e.g. fungi versus arthropod)[17]. The aim of the present study was to use sera from a large group of horses from France, Switzerland, USA

and Canada, exposed to a wide range of potential allergens in the normal equine environment and determine if a combination of microarray and mathematical modelling could be used to elucidate previously unidentified allergens involved in the aetiology of sEA, as a potential diagnostic test for sEA, and to evaluate the influence of samples from mixed environments without matched controls. To achieve this, we primarily used specific allergen molecules, thus identifying genuine sensitisation and minimising cross-reactivity, potentially enabling precise allergen selection for future immunotherapy.

Materials and methods

Equine sera samples

Horses from Canada, France and the US were classified according to clinical assessment, including physical examination, tracheal mucus, pulmonary function test, reversible airway obstruction after medical/environmental change and BALF cytology, demonstrating moderate to severe neutrophilia (>25% cells), as previously described[16]. Control horses had no record of lung disease, no previous history of laboured breathing, coughing or nasal discharge, no tracheal mucus, and <10% BALF neutrophils. Swiss samples were those published in Verdon et al., (2018)[18], sEA was classified using the horse owner assessed respiratory signs index (HOARSI) ≥ 3 and partial pressure of arterial oxygen < 90 mm Hg, and Insect Bite Hypersensitivity (IBH) classified via IBH scoring[18]. Blood was collected from the jugular vein in VACUETTE Serum Clot Activator Tubes, centrifuged at 2000 × g for 10

minutes, serum removed and stored at −80°C. This study was approved by the Royal Agricultural University Ethical Review Group (see supplementary data). All experiments were performed in accordance with the relevant guidelines and regulations.

In the first part of the study, a sub-group of the total of 138 sports horses, consisting of n=35 environmentally matched samples from France (5 sEA; 6 control), USA (6 sEA; 6 control) and Canada (6 sEA; 6 control) were analysed. These were modelled to enable reliable comparison of samples with matched controls collected from horses in the same environment, thus accounting for any antigenic stimuli associated IgE responses. In order to test the robustness and clinical relevance of the test, in the second phase of the study, microarray analysis was carried out on a larger group, including the aforementioned horses and those from differing environments without matched controls (n=138), consisting of sEA n=33, IBH/sEA n=23, IBH n=24 and control n=58 from France, Switzerland, USA and Canada. Horses suffering with IBH, a classic equine hypersensitivity, were included to further assess the discriminatory power and clinical relevance of this approach. This group (n=138) was used to build and test the mathematical predictive model and identify relevant allergens.

IgE sera determination by protein microarray

The comprehensive complex microarray comprised of extracts (n=153) and pure proteins (n=231) from a wide range of fungi, bacteria, pollen, arthropods and others associated with the equine environment. The extracts and pure proteins were obtained from commercial suppliers, produced in-house and donated to our group. Fungi and bacteria strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, grown in liquid media, and extracts produced via sonication. Samples were normalised to 0.5mg/ml protein and printed onto ONCYTE® NOVA Nitrocellulose Film Slides (Grace Bio-Labs, Oregon, USA) using an Ultra Marathon II by Arrayjet, (Roslin, Scotland) to a final spot density of 12,288 spots/slide, with an approximate spot size of 200 µm diameter and replicated twice into two blocks on each pad. For alignment purposes Cy3/Cy5 were included, and for quality control purposes a number of sham antigens were spotted (e.g. PBS, Equ c 3, Ara h 1-NT, Man e, Gal d 1-4). Slides were blocked in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA) at 37 °C for 3 h, washed three times for 2 min in PBS containing 0.05% (w/v) Tween-20, followed by five times 1 min washes with Milli-Q water, and dried via centrifugation (MSE Mistral 3000i, Sanyo, UK) 300 × g for 10 min at room temperature.

Slides were fitted with Proplate slide modules (Grace Bio-Labs, product # 204862) and washed three times (60 second dwell time) with PBST (0.2%). Sera samples were diluted 1:2 with 4% BSA in 0.4% PBST, and 100 µl of prepared sample was added to each well, excluding well 4, which was a

control filled with 100 µl of the dilution solution (1:2) in 2% BSA in 0.2% PBST (final dilution). The Proplate was fitted with an adhesive seal strip and incubated for 16 hours at 4°C on the Stuart mini see-saw rocker (SSM4) at 13 oscillations / minute. Slides were washed three times with PBST (0.05%) using the BioTek plate washer and incubated for 2 hours at 37°C in a ThermoHybaid (HyPro 20) at AVS 3 with 100 µl per well of anti-horse IgE (BioRad, #MCA5982GA) 1:400 in 1% BSA in 0.2% PBST. They were washed a further 3 times with PBST (0.05%) and incubated for one hour at 37°C in the ThermoHybaid with 100 µl per well of DyLight 649 conjugated anti-mouse IgG (Rockland, Product #610-443-040) 1:400 in 1% BSA in 0.2% PBST. Slides were then washed three times with PBST (0.05%) followed by three washes with Milli-Q water and dried via centrifugation at 300 x g for 10 mins (Mistral 3000i, rotor 43124-708).

Data analysis

Processed slides were scanned in a GenePix 4000B (Molecular Devices, USA) with the PMT settings 440 and 310 at 635 and 532 nm and saved as TIF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. Digital fluorescence units (DFUs) were calculated for each spot by subtracting local background from the median fluorescence value of the spot. One pad on each microarray was used as a control, containing reagents and no serum, the results of which were subtracted from all other pads to account for any auto-fluorescence or non-specific binding. Clinically healthy and IBH horses were used as control.

PLS toolbox (version 5.8.3, Eigenvector Research Inc., USA) running on a MATLAB platform (MathWorks, Cambridge, UK) was used to carry out principal component analysis and partial least squares discriminant analysis (PLS-DA) was used as a classifier which enabled construction of the predictive mathematical models[8]. Partial Least Squares Discriminant Analysis (PLS-DA), a type of PLS regression against a dummy matrix, was used to separate pre-defined classes of samples (i.e. affected/non-affected horses). The model was used to inform which specific variables (allergens) are important to determine class prediction[19]. A variable influence on the projection (VIP) score of each variable was calculated as a weighted sum of the squared correlations between the original variable and the PLS-DA components. This is a measure of the contribution that a specific variable has on the model[20]. In order to test the mathematical model produced, multiple rounds of cross validation (CV) were performed using different partitions, and the validation results were amalgamated through the rounds giving an estimate of the model's predictive performance[20].

Results

Environmentally matched group

The initial calibration of the PLS-DA classification method using the small subset of environmentally matched samples (n=35) was highly encouraging, with CV values confirming good prediction (table 1). In an effort to reduce the background noise and improve robustness of the mathematical model, a second round of modelling was conducted using the main VIPs (n=129)

identified in the calibration step (figure 1). This improved both sensitivity and specificity of the (CV) mathematical model (table 1).

Table 1: Partial least squares discriminant analysis statistics of the calibrated and cross validated data from the environmentally matched group of horses (n=35) from the first (before VIP selection) and second (after VIP selection) rounds of modelling. CAL= calibration; CV= cross validation.

	Before VIP selection		**After VIP selection**	
	CAL	CV	CAL	CV
Specificity	1.00	0.70	1.00	1.00
Sensitivity	1.00	0.72	1.00	0.94
Error	RMSEC=0.092	RMSECV=0.480	RMSEC=0.052	RMSECV=0.275

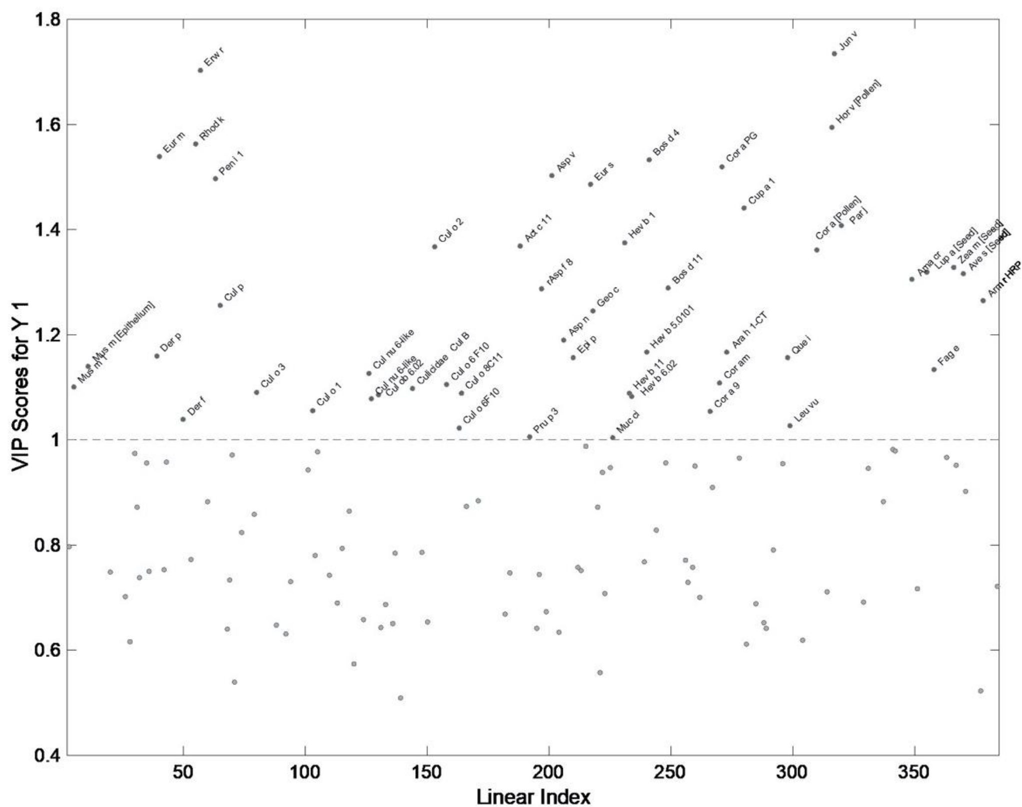

Figure 1: Variable influences on the projection calculated by PLS-DA software from the environmentally matched group of horses (n=35) after VIP selection. A threshold of α>1 was used to identify those VIPs significant in class prediction.

Environmentally mixed group

The mathematical calibration model with the reduced number of sEA horses and matched samples has shown that it is feasible to discriminate sEA from control animals. Whether other allergic diseases and samples without matched controls would interfere with the detection and classification method

has been tested using a cohort of 138 horses (34 sEA, 23 IBH/EA, 23 IBH and 58 controls). The PLS-DA calibration modelling involving this new cohort (n=138) confirmed the good prediction for sEA obtained with non-matched samples, particularly after the second round of mathematical modelling using the sEA VIP selection (table 2). A wide range of VIPs were identified as significant for class prediction (see supplementary data), predominant variables included Hev b 11, Hev b 6.02, Hev b 5.0101, rAsp f 8 and Hel as 7.

Table 2: Partial least squares discriminant analysis statistics of the calibrated and cross validated data from the environmentally mixed group of horses (n=138) showing different classification values after sEA VIP selection. CAL= calibration; CV= cross validation.

	sEA		**Control**		**IBH**	
	CAL	CV	CAL	CV	CAL	CV
Specificity	0.865	0.865	0.787	0.725	0.957	0.902
Sensitivity	0.765	0.735	0.931	0.81	0.826	0.674
Error	RMSEC =0.315	RMSECV =0.360	RMSEC =0.366	RMSECV =0.413	RMSEC =0.108	RMSECV =0.366

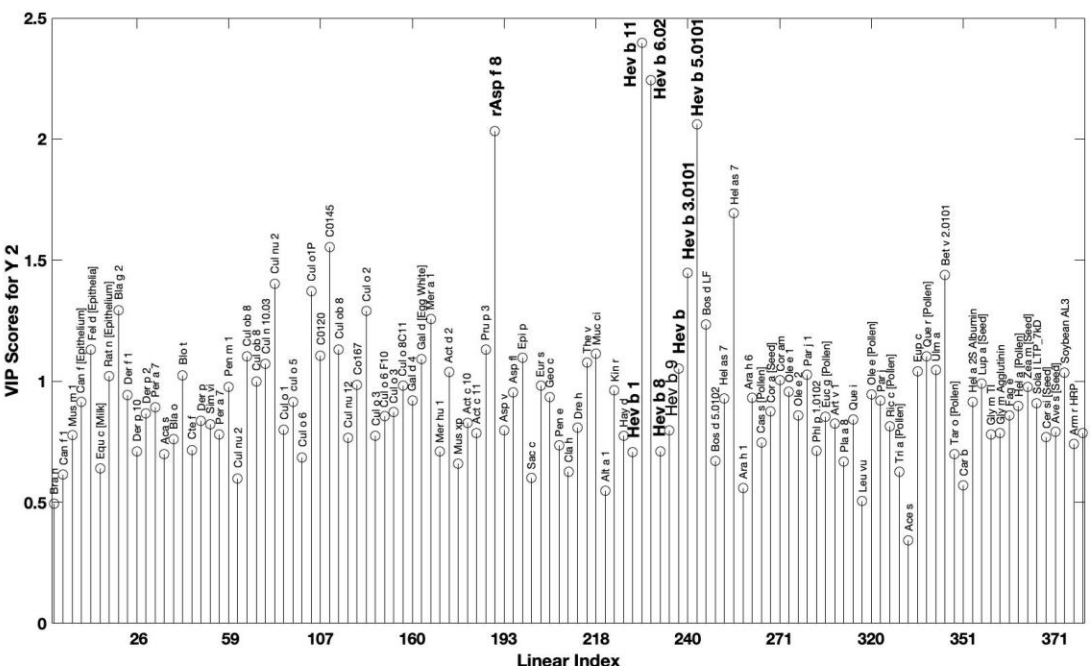

Figure 2: Variable influences on the projection (VIP) scores calculated by PLS-DA from the environmentally mixed group of horses (n=138) after VIP selection. A threshold of α>1 was used to identify those VIPs significant in class prediction.

Discussion

Previously, using Partial Least Squares Regression we demonstrated that human IgE microarray analysis with extracts and pure proteins correlate well with standard laboratory methods such as ELISA, UniCAP and immunoblot test[21]. Similarly, there was good agreement between equine IgE microarray and ELISA results in this study (see supplementary data). Mathmatical modelling of profiling data for disease classification and allergen identification is well utilised in human alogology, but has had little application in the

veterinary sector[8]. Based on these principles, we utilised latest technological developments and mathematical modelling to explore sEA. This enabled the widest scale sEA-associated allergen profiling to date. This study utilised whole protein extracts to maximise allergen coverage, while maintaining specificity by including purified proteins where allergens were known. This was essential because of the limited numbers of potential sEA allergens screened by others to date. Further work would benefit from purifying proteins of the identified whole extracts of interest, thus providing well-defined reagents for component resolved diagnostics enabling increased specificity and sensitivity, particularly aimed at the use of specific immunotherapy[22].

Many of the allergens identified in the initial model (figure 1) have previously been implicated in human allergic asthma, but not previously assessed in the horse. *Erwinia, Geotrichum candidum* and *Eurotium amstelodami* have been associated with asthma and occupational respiratory diseases in farmers[9,23–25]. Moreover, *Junioerus virginiana* and *Corylus avellane* pollen are often noted as inciting hay fever and asthma[26]. Particularly noteworthy allergens include *Aspergillus versicolor*, *A niger* and *A fumigatus* all from the most significant genus associated with the aetiology of human asthma and sEA[25]. Similarly, *Dermatophagoides farinae* is associated with human asthma as well as sEA[12,27], and Pen i 1 is cross-reactive to many arthropods[28]. Furthermore, several latex allergens are significant for class prediction, a group previously untested in the horse.

The classification results (CV sensitivity and specificity) from the second model (figure 2) were however lower than the first small subset of horses. The

discrepancies between the two subgroups most likely results from the second group being collected from varying environments as described by Eder et al., (2001). These authors assessed 450 horses from 6 different yards and concluded stable-specific environments have a highly significant effect on allergen-specific serum IgE levels. The second group presented here did not have matched controls to account for environmental-associated IgE production and possessed a strong IBH response bias which may have weakened the mathematical predictive model. The most influential VIPs for class separation were those from natural rubber latex (Hevea brasiliensis, Hev b), these included Hev b 11; Hev b 6.02; Hev b 5.0101; Hev b 3.0101 and Hev b-extract (see supplementary data). To the authors knowledge, this is the first time Hev b allergens have been assessed in relation to sEA. As shown in Figure 2, a smaller level of IgE-binding to latex allergens was detected in the sera of IBH positive horses and controls used in this study (latex means: 1445, 735, 803 for sEA, IBH and control respectively with $P<0.0001$ when compared to sEA), however latex allergens alone were not able to discriminate the sEA group. As shown in table 2 this discrimination is much improved with the other VIPs, particularly *Aspergillus* (Asp f 8).

Work in human asthma patients has revealed a higher frequency of Hev b allergies in affected individuals[29]. A major source of respirable Hev b allergens in the horse's environment is from artificial riding surfaces. Although the use of recycled tyres was banned in many parts of Europe in 2007, in the UK it is permitted under current Environment Agency waste regulations (Waste Exemption: U8 use of waste for a specified purpose), and many arena

surfaces throughout the world contain components of natural rubber. These surfaces have high levels of respirable dust, which has previously been associated with chronic bronchitis in riding instructors[30–32]. Respiration of Hev b particles have also been shown to induce inflammation and oxidative stress in the lungs of humans[33]. Furthermore, particles, such as Hev b, have been shown to exhibit an adjuvant effect by increasing the primary response during sensitisation when present either before, during or after allergen exposure[34]. Diaz-Sanchez et al., (1999) demonstrated particulate inhalation during allergen exposure could induce a mucosal IgE response under conditions in which the allergen alone could not[35]. Similarly, experimental animal models in strains of mice not prone to developing IgE responses, demonstrated that particulate antigens may enhance sensitisation[36]. Given the adjuvant and sensitising effects of latex, these airborne particles could contribute to the increase in both latex sensitisation and asthma through direct and indirect mechanisms[37,38], which may explain the association between sEA and Hev b-specific IgE demonstrated here. Moreover, these results are in agreement with previous work identifying the urban environment, which is high in respirable natural rubber latex[38], as a risk factor in sEA[39]. In humans, regular exposure to latex particles in the work environment can lead to occupational asthma, commonly known as latex-induced asthma. The prevalence of latex sensitisation in occupationally-unexposed groups is significantly lower (<1%) than those regularly exposed (>18%)[40]. The main allergen associated with occupational latex-allergy (Hev b 6.02)[41] was the second most influential VIP in our study group with sEA-affected horses, along with other major Hev b-allergens used for occupational latex-allergy diagnosis (Hev b 11; Hev b

5.0101)[42]. The results of this study would suggest there may be an association between sEA and increased latex-specific IgE. Further equine specific work is required to establish the exposure levels of latex in the horse's daily environment, demonstrating the benefit of latex avoidance, latex inhalation reactivity tests, epidemiological studies and further hypersensitivity confirmation through basophil activation tests. At present, exposure should be considered a potential risk to the respiratory health of the horse.

Several fungal allergens were found to significantly influence class prediction, these included *Aspergillus fumigatus* (rAsp f 8), *Mucor circinelloides f. lusitanicus* (Muc ci), *Geotrichum candidum* (Geo c) and *Eurotium amstelodami* (Eur a) (see supplementary data). The rAsp f 8 results confirm those of Eder et al., (2000) and Künzle et al., (2007) whom also found significantly more IgE against this recombinant mould allergen in sEA-affected horses[10,11]. Tahon et al., (2009) also reported significantly higher positive intradermal reactions to rAsp f 8 in sEA-affected horses[44]. *Mucor circinelloides f. lusitanicus* (Muc ci) results further confirm previous research demonstrating *Mucor* allergen extract sensitisation is associated with sEA-affected horses via *in vitro* basophil assay[5]. Similarly, increased levels of specific IgE against *E. amstelodami* and *G. candidum* have been identified in the bronchoalveolar lavage fluid of sEA affected horses via western blot[9]. Several arthropods were significant for class separation, including the tropomyosin's of *Helix aspersa* (Hel as 7 and *Periplaneta Americana* (Per a 7), the proteases from *Blattella germanica* (Bla g 2) and *Dermatophagoides farinae* (Der f 1), the complex mixture of *Blomia Tropicalis* (Blo t), and *Dermatophagoides pteronyssinus*

(Der p 2). The array results therefore ratify recent reports on the involvement of *Acarus siro*, *Dermatophagoides, farinae/pteronyssinus, Tyrophagus putrescentia* in sEA and their association with high concentrations of specific IgE against mites, particularly *T putrescentia*[12]. Bla g 2 is associated with the development of asthma in humans and increased sIgE against Bla g has previously been reported in sEA-affected horses[17,45]. Tropomyosin results (Hel as 1 and Per a 7) are to be expected, as Tropomyosin are major allergenic components accounting for cross-reactivity with mites and other arthropods[46]. Furthermore, the high VIP scores demonstrated for *Cullicoides* proteins (Cul nu 2, CO145, Cul o 2) could have resulted from the sEA/IBH horses, even though these were matched with IBH controls, or from multiple hypersensitivities, as sEA horses are at increased risk of IBH[47] which is associated with airway hyperreactivity[48]. The only bacteria considered significant for class separation was *Thermoactinomyces vulgaris*, which has long been associated with sEA and increased levels of IgE in affected horses [49–51]. Interestingly, our study showed 28 pollens were significant for class separation, including *Betula verrucosa* (Bet v 2.0101), *Mercurialis annua* (Mer a 1), *Eupatorium capillifolium* (Eup c), *Quercus robur* (Que r) and *Helianthus annuus* (Hel a). To the authors knowledge, this is the first study to show an association between sEA in horses and a hypersensitivity to pollens. When utilising a panel of 131 allergens, Einhorn et al., (2018) demonstrated that horses are most likely to be sensitised to Fag e 2, Cyn d 1 and Aln g 1, similarly here we found Fag e was significant for class prediction (figure 2)[52].

As expected, the environmentally matched (MA) group has several VIPs in common with the environmentally mixed (MI) group. Most notably Hev b 11,

Hev b 6.02, rAsp f 8, Eur s and Hev b 5.0101. Moreover, many similarities are apparent, such as Der f and tropomyosin Pen i 1 in MA compared with Der f 1 and tropomyosin Hel as 7 in MA. The MI group was equally reliant on a range of aspergillus species (Asp v, Asp n, rAsp f 8), whereas the MA group primarily relied on rAsp f 8. Bovine milk proteins are important for class prediction in both models (MA - Bos d 4, Bos d 9; MI - Bos d LF), the significance of this warrants further research, these molecules commonly cross react between species and have shared common allergenic components with other allergens, such as Glycine max[53].

Use of a PLS-DA model enabled the classification of sEA-affected horses using IgE as a biomarker, which has previously not been possible with the utilised statistical methods due to overlap between affected and non-affected groups[10,52]. Such models have been employed in the human sector to enable diagnosis of asthma patients using metabolomics with great success, and proved to be just as effective with sEA[20]. Furthermore, the identification of specific IgE auto-reactivity through VIP identification contributes to an understanding of the pathogenesis of the disease. The ability to discriminate sEA-affected horses from other IgE-mediated conditions demonstrates the robustness of the test. Further research expanding the repertoire of allergens tested in the form of pure proteins would increase the diagnostic accuracy of the mathematical model as well as benefiting identification of genuine sensitisation and enabling therapeutic and diagnostic development. This advanced bioinformatics enabled the largest scale allergen profiling of sEA to

date, significantly contributing to aetiological understanding of this complex disease.

In conclusion, the microarray platform demonstrated here may be utilised as axillary diagnosis for sEA, informing accurate allergen-avoidance regimes based on its sensitisation profiles; while simultaneously elucidating important factors associated with the aetiology and pathogenesis of this complex disease. Moreover, it enables further diagnostic developments and the creation of specific immunotherapy treatments. This serological investigation of 138 horses living in varying environments identified that sEA is associated with a large sensitisation profile, and predominantly involves latex, fungi, mite and pollen proteins; demonstrating similar profiles to that found with allergic asthma in the human. These results indicate that exposure to latex may be detrimental to the respiratory health of the horse. Further research is required to establish the levels of latex exposure in the equine environment and its *in vivo* effects. Sensitivity and specificity values confirmed the high discriminatory power of the technique in combination with mathematical modelling. The microarray platform demonstrated here will enhance the health, welfare and performance of sEA affected horses. This has been achieved on a number of levels through (a) the development of a novel serological diagnostic test, (b) improved understanding of disease pathogenesis, and (c) identification of novel allergenic candidates.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Acknowledgments

This work was supported by the Morris Animal Foundation (grant no.: D16EQ-039), the Swiss National Science Foundation (grant no.: 310030-160196/1), Stiftung Forschung für das Pferd, the Royal Agricultural University, the Fred

and Marjorie Sainsbury Charitable Trust and HAYGAIN. The authors would like to thank the *Equine Respiratory Tissue Bank for the supply of Canadian samples.*

Author information

Contributions

SW, MA, EM, MMC and DH designed the project. SW, EM, VG, LC and ER contributed to sample collection and diagnosis. SW and MA performed data analysis and processing. SW prepared the first draft of the manuscript. All authors revised the manuscript.

Competing interests

The authors declare no competing interests.

6.0 Paper III

Hypothesis: The thermal treatment of hay reduces IgE-protein binding.

The effect of hay treatments on IgE-protein binding in horses with severe equine asthma via protein microarray profiling: a proof of concept study

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Keywords: *severe equine asthma; horse; protein microarray; forage treatment.*

Abstract: The creation of an accurate and comprehensive test to assess the levels and diversity of severe equine asthma (sEA)-associated allergens present in hay samples is critical for allergen-avoidance regimes, the cornerstone of effective treatment. Previously, White *et al.,* (2019a,b) demonstrated the ability to use microarray platforms in the diagnosis of sEA, as well as, its specificity in allergen identification when using a competition technique (protein inhibition). Here we have demonstrated the ability to use

the same microarray platform to identify sEA-associated allergens in hay samples, and assess the effect soaking and steaming has on IgE-protein binding. Horses (n=3) from three different yards were analysed in conjunction with their corresponding hay samples after several treatment methods (dry; soaking; steaming). There were no significant differences in IgE profiles between hay treatment methods, demonstrating that these forage preparation techniques have no influence in IgE-protein binding. This technique enabled evaluation of environmental samples, and can be used to inform tailored allergen-avoidance programs.

1.0 Introduction

Severe equine asthma (sEA, heaves) is a prevalent, performance limiting disease, initiated by an allergic response to the respirable airborne allergenic proteins present in organic dust in genetically predisposed individuals (Halliwell et al., 1993; McGorum et al., 1998). Although the pathogenesis of sEA remains unclear, in this context the cross-linking of IgE and specific aeroallergens initiates the inflammatory reaction (Moran et al., 2010a,b; Schmallenbach et al., 1998; Halliwell et al., 1993; Eder et al., 2001; Curik et al., 2003; Kunzle et al., 2007). The most prolific sources of organic dust in the horse's environment are hay and straw bedding, which are rich in pollens, mite, bacteria and moulds (Bogacka and Jahnz-Rózyk, 2003; Künzle et al., 2007; Moran and Folch, 2011; Pirie et al., 2003; Tahon et al., 2009).

Many horses are stabled in an environment that presents a profound aeroallergen challenge to the respiratory system. Hay exposure in the horse's early life is a risk factor for the later development of sEA (Hotchkiss et al., 2007a). Despite this, hay is fed by 69.3% of owners over the winter period, 40.7% of which is soaked (26.8% not fully immersed) (Hotchkiss et al., 2007; Robinson, 2001; Vandenput et al., 1997).

The proliferation of potential sEA-associated inciting allergens, including bacteria, mites, mould and their metabolites, often occurs during storage as a direct result of conserving hay with a moisture content in excess of 20% (Gregory et al., 1963; Séguin et al., 2012; Terho et al., 1982). Many of these microorganisms are on the crop when harvested, so even well conserved hay can contain a large range and number of sEA-associated allergens. As a result, hay is one of the major contributors to respirable sEA inciting antigens in the stabling environment (Webster et al., 1987).

Hay dust inhalation challenge in sEA horses results in airway neutrophilia, obstructive airway dysfunction and mucus hypersecretion, and the severity of the response appears to be dose dependent (Pirie et al., 2002). Furthermore, sEA horses in contact with hay associated proteins demonstrate clinical symptoms within 1.5 hours of exposure, showing significant alteration in their mechanics of breathing and arterial bloods (Halliwell et al., 1993; Vandenput et al., 1998a), when at pasture or fed silage in a stabling environment these parameters were similar to healthy horses (Vandenput et al., 1998b). When evaluating soaked and steamed hay, Blackman and Moore-Colyer (1998)

emphasised the value of hay treatments is not to enable the feeding of hay to hypersensitised horses, but in the reduction of respiratory challenge to healthy horses and to prevent sensitisation to asthma-inducing allergens. More recently, increased attention has focused on the use of thermal processing to reduce allergenicity in human foodstuffs (Verhoeckx et al., 2015), utilising thermal processing techniques like steaming, which similarly has been shown to reduce horse: allergen interaction in horse hay by reducing airborne respirable dust (ARD) (Moore-Colyer et al, 2016). However, the influence of hay steaming on the allergenic potential of proteins remains unclear.

Previously, White et al. (2017) demonstrated the ability to diagnose sEA via protein microarray profiling. This method allowed sensitisation profiling of hundreds of proteins and enabled the identification of a plethora of previously unidentified sEA-associated allergen, primarily moulds, bacteria, pollen and arthropods (White et al., 2017; White et al., 2018; White et al., 2019). Furthermore, competition methods were developed for the microarray to test the specificity of allergen identification via protein inhibition assays, confirming its efficacy in allergen identification within a given sample (White et al., 2017). The aim of this study was to determine the effect current physiochemical hay treatments have on IgE-protein binding in sEA-affected horses using competition microarray.

2.0 Methodology

2.1 Sample collection

2.1.1 Hay samples

Belgium medium-cut meadow hay which had been consumed by the horses for a minimum of three months prior to testing was prepared using the following treatments: 1) dry where no additional treatment was applied; 2) soaked by total immersion in clean tap water for 10 minutes at 16°C as described by Moore-Colyer et al. (2014); 3) steamed in the HAYGAIN 600 hay steamer, for a total of 50 minutes but also ensuring that a minimum of 10 minutes were recorded when the integral thermometer reached 80°C (reflective of an internal hay temperature of 100°C according to the manufacturer). Digi-Sense Irreversible High Temperature Labels were placed on sterile glass rods at the centre of the hay at 25%, 50% and 75% intervals to confirm an internal temperature of 100°C was reached (Propress Equine Ltd, Hungerford, UK).

Post treatment, hay was mixed on a clean sheet and twenty “grab” samples (each yielding approximately 25g) were taken at random across the sampling area in a W-formation. The “grab” samples were pooled into a 1650ml capacity sterile sample bag (VWR, Lutterworth, UK) and stored at ambient temperature for a maximum of three hours out of direct sunlight and prepared for long-term storage by roughly chopping the hay with scissors (Bochem, Weilburg,

Germany) on a laboratory tray (Multiroir-Controlec, Périgny, France), both of which had been cleaned with disinfectant wipes (Vernon Carus, Lancashire, UK). The roughly chopped hay was tightly packed into four 50 ml capacity centrifuge tubes (VWR, Lutterworth, UK), and stored at -80°C until further analysis.

2.1.2 Sera samples

The sample group consisted of three sEA-affected warmblood horses from three separate yards in Belgium. Sera samples were collected as part of a routine clinical sEA-diagnostic assessment and not for research purposes. All horses were fed dry hay and stabled on wood shavings. Severe equine asthma was classified according to BALF cytology, pulmonary function test, clinical assessment and physical examination as previously described (Couëtil et al., 2016). VACUETTE Serum Clot Activator Tubes were used for collection of blood from the jugular vein, centrifuged at 2000 x g for 10 minutes, serum aliquoted and stored at −80°C until analysis, at which time the samples were thawed at room temperature and placed on ice. Experimental protocol was approved by the Ethical Review Group at the Royal Agricultural University.

2.2 Environmental sample protein extraction

Three grams from each of the forage samples was placed in a desiccator with silica gel overnight, once dry, the sample was chopped in a pre-sterilised coffee grinder for 1 minute. 1.5 g of chopped sample was milled using a

Pulverisette 7 (Fritsch, UK) with 12 ml zirconium oxide ball mill bowl and 6 x 10mm balls (500 rpm, 5 minutes milling, 5 minutes pause cycle, reverse cycling, 11 rips). 1g of finely ground sample placed in a 50ml centrifuge tube with 10 glass beads and 5ml of extraction buffer (5ml PBS, 50 µl Tween 20 and 1 Pierce protease inhibitor tablet (product # 13437766), filtered through 0.45 µm syringe filter) and rolled at 4ºC for two hours. Samples were centrifuged in a Mistral 3000i at 2500 rpm, for 10 minutes at 4ºC (Renault *et al.,* 2011), and supernatant removed into an Eppendorf tube and placed on ice. Immediately before use, samples were centrifuged at 12,000 rpm for 30 seconds in Beckman Coulter Microfuge 22R centrifuge.

2.3 In-house prepared extracts

The protein extracts and pure/recombinant proteins were produced in-house, donated or obtained from commercial suppliers (n=384). Strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen and grown in 150ml of liquid media according to the supplier's recommendations, and whole extracts prepared via sonication methods and normalised to 1mg/ml protein in MiliQ water with 10% glycerol using a Pierce BCA Protein Assay Kit (23225) and stored at -80 ºC as previously described (White et al., 2019a).

2.4 Competition microarray

The comprehensive microarray contained pure proteins (n=231) and extracts (n=153) from a wide range of protein families, including pollen, bacteria and

arthropods. Normalised samples (0.5 µg/ml protein) were printed onto ONCYTE® NOVA Nitrocellulose Film Slides (Grace Bio-Labs, Oregon, USA) using an Ultra Marathon II by Arrayjet, (Roslin, Scotland) to a final spot density of 12,288 spots/slide. The samples were printed with an approximate spot size of 200 µm diameter and the same size spacing replicated twice across the slide into two individual blocks. Spots of PBS and diluted streptavidin-Cy3/Cy5 were included for quality control and alignment purposes. Slides were blocked in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA) at 37°C for 3 h, washed three times for 2 min in PBS containing 0.05% (w/v) Tween-20, followed by five times 1 min washes with MiliQ water, and dried by centrifugation (MSE Mistral 3000i, Sanyo, UK) at 300 × g for 10 min at room temperature as previously described (White et al., 2019a).

The forage extract was diluted 1:10 in 4% BSA in 0.4% PBST containing Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free (product # 13437766)(1 tablet in 5ml), which had previously been passed through a Whatman 13 mm, 0.45 µm filter syringe. 50 µl of forage extract was pre-incubated with 50 µl of sera for 1 hour at room temperature (Lin *et al.,* 2006) in a 96 well ELISA plate on a Stuart mini see-saw rocker. One serum sample from each horse was incubated with 50 µl 4% BSA in 0.4% PBST containing Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free instead of hay extraction to identify the sensitisation profile (i.e. levels of fluorescence units indicating sIgE levels for each protein). During this process, the specific IgE in the sera binds with its corresponding allergen if present (figure 1).

The microarray was loaded with the preincubated samples and the Proplate fitted with an adhesive seal strip and incubated for 16 hours at 4°C on the Stuart mini see-saw rocker (SSM4) at 13 oscillations / minute. Slides washed three times with PBST (0.05%) using the BioTek plate washer and incubated for two hours at 37°C in a ThermoHybaid (HyPro 20) at AVS 3 with 100 µl per well of anti-horse IgE (BioRad, #MCA5982GA) 1:400 in 1% BSA in 0.2% PBST. Washed a further 3 times with PBST (0.05%) and incubated for one hour at 37°C in the ThermoHybaid with 100 µl per well of DyLight 649 conjugated anti-mouse IgG[1] (Rockland, Product #610-443-040) 1:400 in 1% BSA in 0.2% PBST. The slides were then washed three times PBST (0.05%) followed by three washes with Milli-Q water and dried via centrifugation at 300 x g for 10 mins (Mistral 3000i, rotor 43124-708).

Processed slides were scanned in a Genepix 4000B (Molecular Devices, USA) with the PMT settings 440 and 310 at 635 and 532 nm and saved as TIF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. Digital fluorescence units were calculated for each spot by subtracting local background from the median fluorescence value of the spot. For control purposes, one pad on each slide contained all reagents but excluded serum, the results of which were subtracted from the samples on the same slide to account for any protein autofluorescence or non-specific binding. Reduction in binding of sera samples following pre-incubation of serum with hay extract indicates that IgE-protein binding occurred during preincubation, therefore the specific allergen was present and recognisable in the sample (see figure 1).

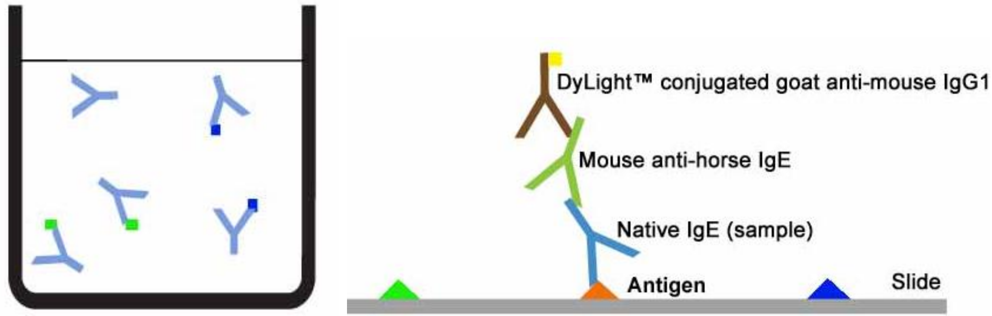


Figure 1: One-hour preinubation with forage extract (left) allows for allergen specific IgE-protein binding in the forage prior to microarray hybridization (right). Preincubation bound IgE will no longer be available for IgE-allergen binding on the array, and is therefore measurable as a decrease in fluoresence units when compared with serum.

2.5 Data analyses

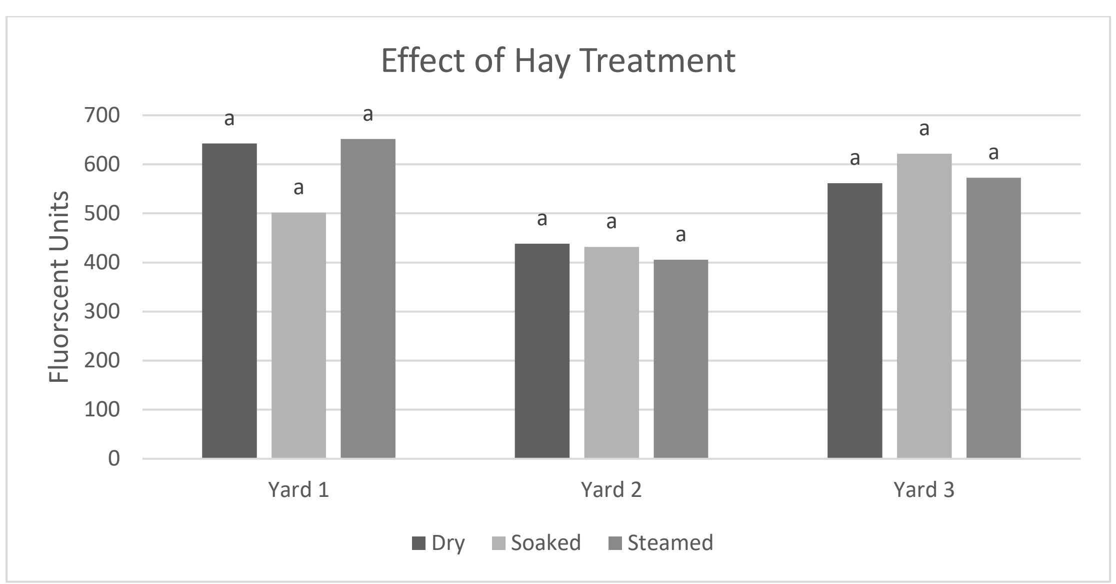
Microsoft Excel was used for data presentation and further analysis. Specific allergen present in the hay were selected and differences between hay treatments (per yard and individual allergen) tested by 1-way analysis of variance. Tukey's honestly significant difference test for multiple comparisons was performed if significant differences were found ($P < .05$).

3.0 Results

3.1 Allergens identified in hay

The methodology adapted from White et al., (2019) inhibition assays enabled the evaluation of allergen presence in varying hay samples. Through the evaluation of altered IgE-protein binding between sera/hay samples and sera alone, a wide range of allergenic genera was identified across all 3 hay samples. The allergen profiles identified (supplementary data) demonstrated clear horse/hay variations, likely a result of differing sensitization profiles between horses and allergens present within the hay sample. Altered IgE-protein binding profiles revealed the most commonly identified allergen across all hay samples was from the genus *Aspergillus* (*A. fumigatus, A. nidulans, A. niger, A. versicolor, A. restrictus*). The most significant arthropod allergen present in hay was *Tyrophagus putrescentiae*, however others included Bla g, Der p 1, Der p 7, Eur m and Lep d. One horse/hay combination identified several bacterial allergens, including *Thermoactinomyces vulgaris,* however this was thought to be highly dependent on the hay samples. Unsurprisingly, several pollen allergens were also identified in hay, including Phl p, Hol l, Lol p1, and Sor h.

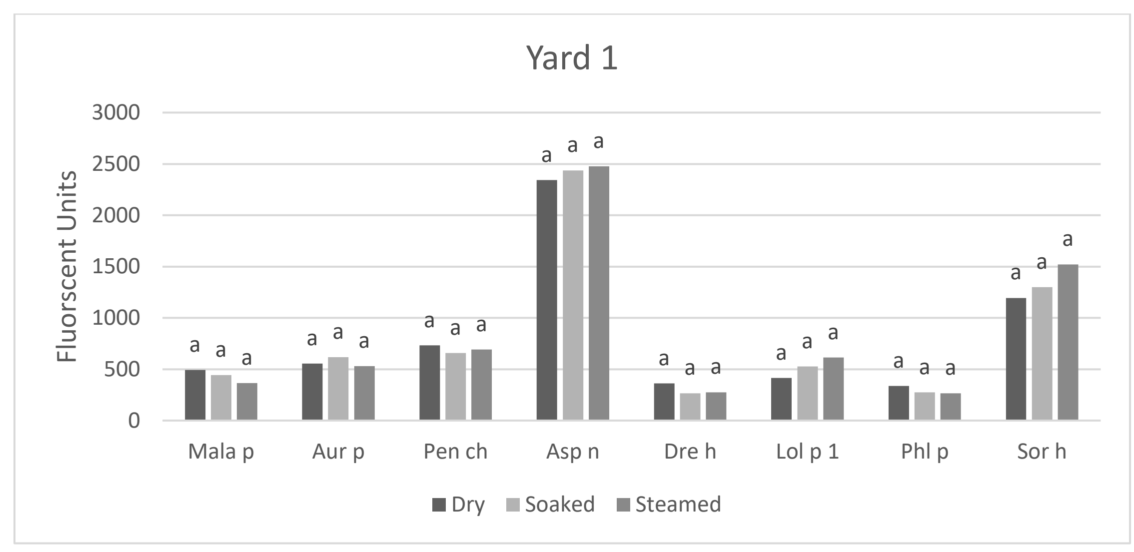
3.2 The effect of hay treatment methods on IgE-protein binding

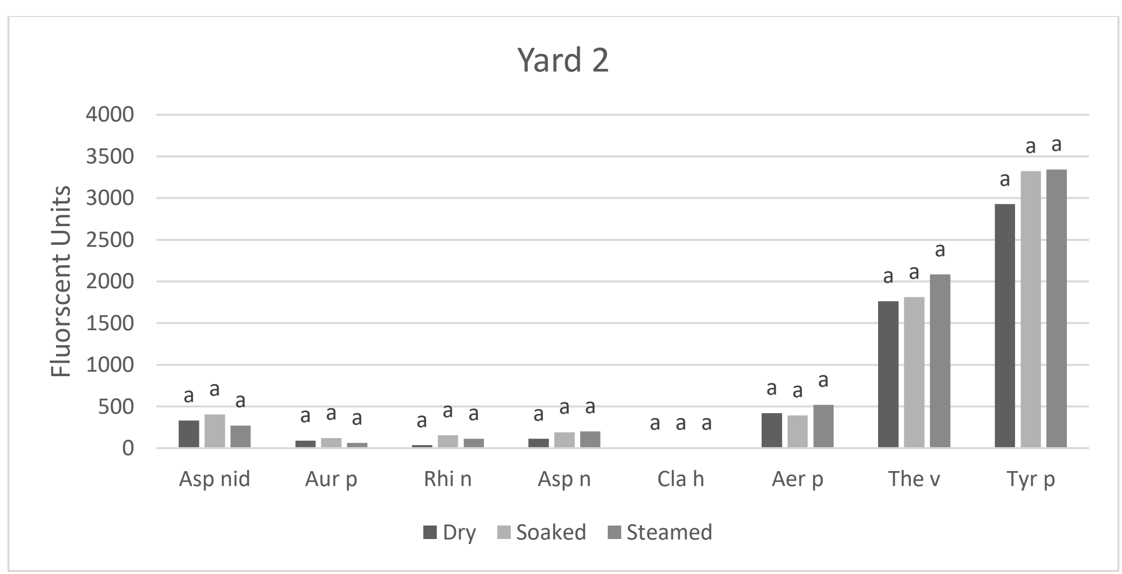


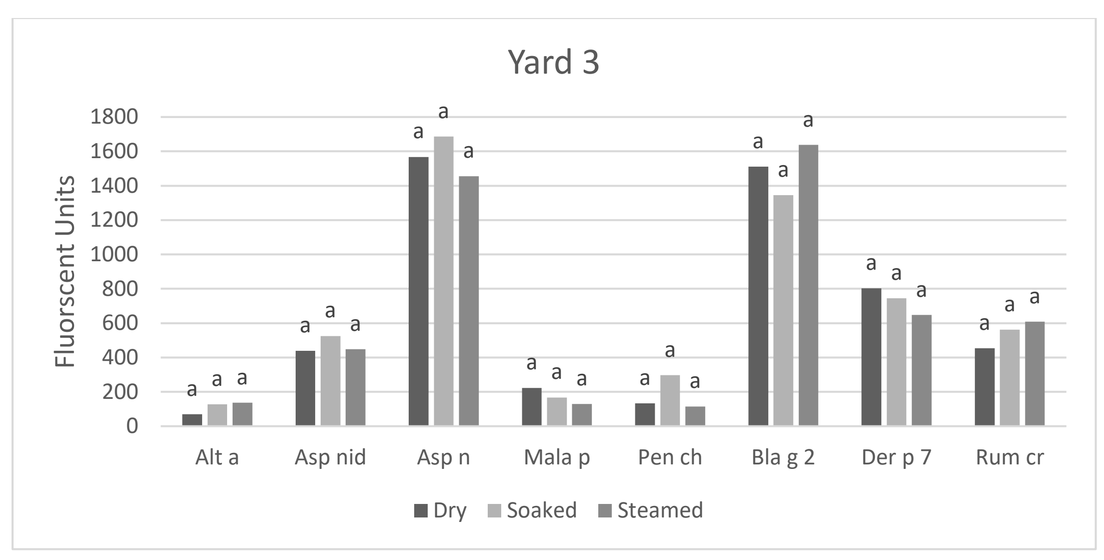
Figures 2: Hay competition experiments using the protein array. Average cumulative fluorescent units of all hay allergen identified in 3.1 for each horse/hay combination and significant differences by one-way ANOVA with post-hoc for each yard. *Means* that have no *superscript* in common are *significantly different* from each *other* (* = p<0.05; ** = p<0.01).

The one-way ANOVAs suggest that the hay treatments are not significantly different for the level of significance, ergo hay treatment methods do not appear to alter IgE-protein binding.

3.2.1 Allergens of Interest







Figures 3 A – C: Competition experiments using sEA specific allergen identified in the hay samples for each horse/hay combination.

Average fluorescent units of specific sEA-associated allergens demonstrating varying hay treatments from each yard and significant differences by one-way ANOVA with post-hoc for each allergen. Each yard represents a single horse-hay analysis. *Means* that have no *superscript* in common are *significantly different* from each *other* (* = p<0.05; ** = p<0.01). *Malassezia Pachydermatis* = Mala p; *Aureobasidium Pullularia* = Aur p; *Penicilium notatum* = Pen ch; *Aspergilus niger* = Asp n; *Helminthosporium spondylocladium* = Dre h; *Rye Grass* = Lol p 1; *Phleum pratenses* = Phl p; *Sorghum halepense* = Sor h; *Aspergillus nidulans* = Asp nid*; Rhizopus nigrican/stolo* = Rhi n; *Cladosporium herbarum* = Cla h; *Aeromicrobium ponti = Aer p; Thermoactinomyces vulgaris* = The v*; Tyrophagus putrescentiae* = Tyr p; *Alternaria alternaria* = Alt a; *German cockroach* = Bla g 2; *Mite* = Der p 7; *Rumex crispus* = Rum cr.

The one-way ANOVAs suggest that the hay treatments are not significantly different for the level of significance, ergo hay treatment methods do not

appear to alter IgE-protein of sEA associated allergen specific to these particular horses.

4.0 Discussion

To date, environmental allergen testing has been confined to testing an environmental sample for the quantity of a specific pre-established allergen using small scale methods, such as ELISA or lateral flow assays, making wide-scale assessment unfeasible. To the author's knowledge, this is the first study to utilise native IgE for the identification of patient-specific allergen in environmental samples utilising a microarray platform. Previously, we demonstrated the efficacy of protein microarrays in the identification of allergens associated with sEA, and the techniques specificity in allergen identification using competition methods (White et al., 2018; White et al., 2017). The refined competition methods for environmental samples described here provided a highly specific test for the analysis of IgE-protein binding of environmental samples, enabling the analysis and evaluation of varying hay treatment methods.

A range of potential allergens have previously been detected in hay, predominantly fungi including, *Aspergillus fumigatus, A. niger*, *A. flavus, A. glaucus, , A. nidulans, Penicillium* spp., *Absidia* spp., *Mucor hiemalis, M. pusillus*, and *Fusarium* (Araya et al., 1995; Buckley et al., 2007; Gregory et al., 1963). Bacteria identified mainly consists of thermophilic and thermotolerant actinomycetes, such as *Thermoactinomyces*

vulgaris and *saccharopolyspora rectivirgula* (Araya et al., 1995; Clarke and Madelin, 1987; Gregory et al., 1963). Mites are commonly identified in hay proportionately to fungal quantity, the most prolific of which are *Lepidoglyphus destructor* and *Acarus siro* (Hage-Hamsten et al., 1991; Hillerdal et al., 1982). The quantity of potentially allergenic proteins assessed in hay to date is limited in contrast to the wide range of sEA-associated allergens previously identified by the authors utilising microarray methods (White et al., 2018; White et al., 2017). Adapting these techniques, it was possible to identify specific allergen sensitisation in a certain animal, and evaluate the hay sample as a specific allergen source. The results here are in agreement with previous literature, hay is a major source of sEA-inciting allergen. Furthermore, the technique was able to demonstrate some instances in which hay was not the predominant allergen source (supplementary data), this could be a result of previous sensitisation resulting from hay from a past season, or from another environmental source, such as bedding or circulating pollen. The influences of differing prior aeroallergen exposure was reflected in the varying sensitisation profiles between yards. Evaluation of the most prominent allergen associated with sEA confirmed hay is a major source of *A. fumigatus,* an allergen ubiquitous to the debilitating disease. Moreover, the technique was able to identify differing sensitisations, such as the pollens for the horse from yard 1, and then identify the specific-allergen and potential source through hay analysis. This technique has been shown to be effective in identifying allergens present in the horse's hay, demonstrating its ability to identify allergens in environmental samples associated with the stabling. The same method could be applied for the assessment of bedding and swab samples of

stables. This technique has great potential in assisting the formulation of effective allergen-avoidance programs. However, it is limited to allergens included on the assay, and the allergens to which the specific horse is sensitised.

Hay treatment methods, such as soaking and steaming, rely on preventing allergen:animal interaction through the reduction of respirable particles (Clements and Pirie, 2007; Ivester et al., 2014). The mechanisms behind soaking and steaming hay are yet to be elucidated, but it is well reported that both methods reduce airborne particulate matter (Moore-Colyer et al., 2014). However, the use of steamed hay as a non-medical therapy for sEA-affected horses has previously been questioned (Orard et al., 2018). *In vivo* assessment of the effect of hay treatments on initiating sEA has been sparse and contradictory to date. Blumerich et al. (2012) demonstrated that feeding steamed hay as opposed to dry hay reduced tracheal mucus scores and clinical signs in sEA-affected horses (Blumerich et al., 2012). However, more recently, Orard et al., (2018) found no significant differences in clinical signs, BALF cytology or cytokine profiles in sEA-affected horses fed dry or steamed hay, although they did report that mucus score was significantly higher after dry hay challenge compared with the steamed hay challenge (Orard et al., 2018). The hypothesis behind this study was that the thermal process of steaming hay could lead to the denaturation of allergenic protein, preventing animal:allergen interaction. There are many examples of reduction in allergenicity by heating, but although heat-denatured epitopes can be destroyed, they can also present new allergenic-sites uncovered in the

unfolding process, and therefore may not be a suitable process to produce hypoallergenic foodstuffs (Davis and Williams, 1998). The impact thermal processing will have on an allergen completely relies on the protein structure and stability, as well as the structure of the feedstuff in which the allergen is present (Mills et al., 2009).

The results of this present study demonstrate that steaming hay in the HAYGAIN 600 does not alter IgE-protein binding, thus confirming the hypotheses by previous researchers that steaming does not significantly prevent the synergistic inflammatory activity of hay dust components (Orard et al., 2018). Further investigation should assess the effect of steaming on protein coagulation which may result in “fixing” allergenic proteins to the hay surface, as some researchers have suggested, unlike soaked hay, steaming reduces fungal particles in tracheal washes (Dauvillier et al., 2018). However, controlled studies have indicated steamed hay may still contain mould and bacterial wall fragments triggering an inflammatory response (Orard et al., 2018). The methods behind which steaming reduces clinical signs appears to be through reducing allergen:animal interaction, rather than any significant alteration in allergenic epitopes. These results demonstrate that hay, even after soaking and steaming, still contains sEA inciting allergens and emphasises the need for further research to elucidate the mechanisms behind hay treatments to elucidate the best methods to prevent allergen:animal interaction.

5.0 Conclusion

The microarray platform described here can be used to assess the presence of sEA-associated allergen in hay, and assist in the creation of allergen-avoidance programs. This technique could have future application in further environmental testing, such as bedding and environmental swabs. These results would suggest that current hay treatment methods have no effect on IgE-protein binding, and preventing allergen inhalation is most effective for sEA affected horses. The novel competitive microarray platform demonstrated here can be employed to enhance the health, welfare and performance of sEA affected horses by enabling accurate environmental allergen testing and the implementation of effective avoidance strategies.

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7.0 Methodological considerations

7.1 Sample collection and preparation

7.1.1 Horses and sites

The use of animals in experiments and testing is regulated under the Animals (Scientific Procedures) Act 1986, which requires licencing of animals, facilities, staff and specific projects. Due to the associated costs, complexities and time required to secure licencing, samples surplus to diagnostic and/or research requirements were obtained for all the studies from our network of collaborators in France, Belgium, Switzerland, USA and Canada.

When selecting samples, careful consideration was given to many aspects that may influence sIgE production. Curik et al., (2003) suggested that there could be a breed variation as there is a genetic influence on specific IgE response to recombinant *Aspergillus* proteins, potentially through the MHC gene region and possibly the MHC class II loci (Curik et al., 2003). Similarly, in other IgE mediated conditions, such as IBH, breed and origin have been shown to also play a role in IgE production, where a marked rise was noted in total IgE levels in Icelandic IBH-affected horse sera samples, compared with non-affected horses, but not in the serum of healthy horses from other breeds indicating differences in IgE production between breeds (Wilson et al., 2006). However, more recent work by Wilkolek et al. (2014) found no significant difference between three horse breeds (Malopolski breed, Primitive Polish horses and ponies). To account for this, we ensured that matched samples

contained control and affected-animals of the same breed from each site, whereas mixed samples consisted of a range of sport horses. There is increasing difficulty in assessing a single breed due to the genetic diversity of the modern competition horse, largely a result of ongoing outcrossing from stallions approved by several studbooks (Petersen et al., 2013). For example, Hamann and Distl (2008) evaluated the genetic diversity of the Hanoverian warmblood, where they demonstrated English Thoroughbreds contributed nearly 35% of their genes to the Hanoverian population.

Environmental conditions also play a key role, with researchers demonstrating that the environment influenced sIgE levels against a range of recombinant mould proteins (Eder et al., 2001). Wilkolek et al. (2014) found elevated levels of sIgE recognising mould in horses that were stabled compared to those that lived out. Similarly, Wagner et al. (2003) found that total IgE concentrations in IBH-affected horses was increased, but not significantly higher than non-affected horses kept within the same environment, suggesting environment can play a key role when evaluating sIgE. To account for this, we selected a range of environmentally matched and environmentally mixed serum sources to further validate the mathematical predictive model (paper II) and to evaluate the influence of environments. Research by Wilkolek et al. (2014) has indicated that season does not exert a significant effect on sIgE and neither does age (Kunzle et al., 2007).

A single research herd from the USA consisting of n=6 control and n=6 sEA was originally used during the development stage (paper I) to prevent

environmental variations and influences affecting optimisation (Eder et al., 2001). These horses were clinically assessed (including BALF, pulmonary function tests and physical examination) and diagnosed by a single clinician. In the wide scale assessment (paper II), the 138 horses came from several different regions; controlled samples were collected from Canada, France and the USA, and were classified by a separate clinician at each site, performing a clinical assessment, including physical examination, pulmonary function test and BALF cytology as previously described (Couëtil et al., 2016). Control horses had no previous history of coughing or nasal discharge, no tracheal mucus, and <10% BALF neutrophils; sEA horses were diagnosed on clinical investigation, reversible airway obstruction and BALF cytology, demonstrating moderate to severe neutrophilia (>25% cells). The Swiss samples were those published in Verdon et al., (2018). Severe equine asthma was classified using the horse owner assessed respiratory signs index (HOARSI) ≥ 3 and partial pressure of arterial oxygen < 90 mm Hg, and Insect Bite Hypersensitivity (IBH) classified via IBH scoring (Verdon et al., 2018). HOARSI is a common and well validated technique that correlates with clinical and cytological findings (Rettmer et al., 2015).

BALF was collected surplus to diagnostic or research requirements from the Belgium, French and American horses (paper I and III). As collection was conducted by a different clinician at each site, we took measures to ensure it was as standardised as possible. BALF collection can be performed in a variety of ways, and often varies between clinicians, the most substantial of which is instilled volume of saline. Sweeney et al. (1992) found that cytological

values in the BALF of healthy horses varied considerably and this was the result of a failure in following standardised protocols for quantities of fluids infused (Sweeney et al., 1992). Previous cytological work has shown one lung may not be representative of findings in the contralateral lung of the same horse, but more recently Hermange et al., (2019) demonstrated good agreement between the diagnostic reliability for the final diagnosis of equine asthma. Moreover, consideration should be given to IgE-allergen binding in BALF prior to analysis as a result of inhaled allergen, potentially skewing the allergen profile and impeding BALF x sera comparisons.

7.1.2 Bronchoalveolar lavage fluid

When preparing BALF samples consideration must be given to ensure the preservation of immunoglobulin. Firstly, immediately after aspiration from the lung, samples were filtered (paper I) to remove debris, a common practice when preparing BALF samples for Ig analysis (Ainsworth et al., 2002). Mucus and large cells are often removed from BALF samples via centrifugation (Ainsworth et al., 2002) or through a monolayer of surgical gauze (Künzle et al., 2007), centrifugation is often the preferred method, and does not affect total cell counts (Lapointe et al., 1994). As we needed to prepare samples on site and wished to keep them as sterile as possible, we used frit filter syringes to immediately remove mucus and large cells after aspiration.

After this, protease inhibitor was immediately added as previously reported (Ainsworth et al., 2002). Some researchers have opted not to use protease

inhibitors and merely store samples on ice (Künzle et al., 2007), however, the lung and subsequent BALF contains a wide range of protease (Hunninghake et al., 1979). Proteolytic cleavage, the process of breaking the peptide bonds between amino acids in proteins of immunoglobulin is well reported (Chapman and Mitchell, 1982; Rauter et al., 2008). As such, it was felt protease inhibitors were essential to prevent proteolysis, and ensure the immunoglobulin was viable for analysis (Granelli-Piperno and Reich, 1978).

Similarly, immunoglobulins in BALF are greatly affected by freezing, the protein structure can be destroyed and rendered ineffective as a result of ice crystals forming. For this reason, ethylene glycol or glycerol can be used as a cryopreservative, reducing the aqueous solution's freezing point by stopping hydrogen bond formation between water. As such, glycerol was added to the BALF as a cryoprotectant to a final concentration of 20% to help stabilise the proteins, and prevent formation of ice crystals at -20°C that destroy protein structure (paper I) (Pinsky et al., 2003). Similarly, freeze-thaw cycles, are well known to have little effect on the immunoglobulin present in serum, but this effect has not been well researched in bronchoalveolar lavage fluid (Castro and Jost, 2013; Rastawicki et al., 2012). As such, BALF was subsequently aliquoted prior to freezing to prevent unnecessary freeze-thaw cycles.

Bronchoalveolar lavage fluid samples have been tested without concentration (Wilson et al., 2002), and Peeters et al., (1984) which indicated that developing technology means the sensitivity is such that many immunoglobulin isotypes in BALF can be assessed unconcentrated to the nanogram or microgram.

Furthermore, this has also been demonstrated in previous equine IgE work (Halliwell et al., 1993a; Schmallenbach et al., 1998b). Conversely, Kunzle et al., (2007) found that it was not possible to detect specific IgE in BALF by ELISA, as BALF is too dilute; moreover, after concentration using various techniques, it was still not possible to detect sIgE in BALF. They also concluded that concentrating BALF most likely led to denaturation of the epitopes. In initial trials utilising ELISA tests, this study found that it was not possible to determine allergen-specific IgE levels in BALF, which was further confirmed through microarray profiling and was also in agreement with other studies (Eder et al., 2000b; Wilson et al., 2002). Previously, BALF had most commonly been concentrated 10-fold prior to sIgE analysis, enabling accurate analysis and profiling (Bartlett et al., 2013; Peebles et al., 2001); however, some researchers analysing cytokines have concentrated BALF up to 100-fold (Maitra et al., 2012; Koh et al., 2001). In initial trials in this study a 10-fold dilution was utilised, however, due to the general low-level of fluorescent units at this concentration, further optimisation was necessary, ending with a 40-fold final concentration (paper I).

Several concentration techniques have previously been utilised to concentrate BALF immunoglobulin for analysis in humans, the most common being lyophilisation (Pringle et al., 1998; Wilkie and Markham 1979) as well as centrifugal filtration methods (Peebles et al., 2001; Koh et al., 2001). Other methods have been reported with varying success, but care must be given in selecting a concentration technique, as some methods, for example ammonium sulphate precipitation, may cause denaturation of the labile

epitope(s) (Marti et al., 1997). Lyophilising, also known as freeze drying, is a low temperature dehydration method in which the product is frozen, and pressure lowered to remove ice by sublimination. A major concern was lyophilising without desalting the sample would substantially increase sodium chloride (NaCl 9%, when using NaCl 0.9% saline and performing 10 x fold concentration). Although a figure of above NaCl 20% is often quoted to denature proteins, a concentration of 9% has the potential to damage immunoglobulin, and to denature the antigen extracts present in the immunological assay. For these reasons, when lyophilising it is common to desalt the sample via dialysis, prior to lyophilising and then reconstitute to the required dilution (Pringle et al., 1998; Markham, 1979). However, performing dialysis or a buffer exchange can result in immunoglobulin losses. This added further complexity as the BALF samples in paper I were to be stored frozen and contained a cryopreservative (20% glycerol). Samples containing cryopreservative have a tendency to thaw during lyophilisation, ergo, it was not possible to achieve the desired concentration using lyophilisation. To remove excess salt and the cryopreservative, PD-10 desalting columns (GE Healthcare, 17-0851-01) were used prior to lyophilisation. PD-10 columns have been extensively used in antibody purification protocols due to their ease of use and high antibody recovery. PD-10 does however have several drawbacks including the increased risk of antibody contamination, and difficulties in regulating temperature during the desalting process. For this reason, it was decided to opt for the simpler and widely utilised method of centrifugal filtration, the most readily used of which is Amicon Ultra-15 (previously known as Centricon-10). Some authors opt to include protease

inhibitors and occasionally perform a buffer exchange via dialysis prior to concentration (Bartlett et al., 2013; Peebles et al., 1998; 2001; Lamkhioued et al., 1997; Richard et al., 2014; Foster et al., 2013). The use of a centrifugal filter generally results in a 20% loss of specific IgE and IgG (Peebles et al., 1998; Merck Millipore, 2017). This method has been successfully used to assess specific IgE, IgA, IgM and IgG antibodies (Peebles et al., 2001).

The concentration methods trialled in paper I appeared to show a bias to pollen concentration. In humans and other animals, the responses to pollens are always amplified. Moreover, the strong allergic power of several pollen allergen is linked to their high solubility, which is a relevant factor in the induction of the Th2 response (Huang et al., 2017). This might be due to the extensive polyploid that takes place in plants. By domestication or natural selection, one finds too many gene duplications, hence a high number cross react between the species. There were a large number of pollen spots (n=76) in this study and this definitely might have biased the results. Despite this there was good correlation between the BALF / serum, although the strength of correlation appeared to be partialy dependent on allergen source (fungal vs bacteria vs pollen vs arthropod).

7.2 Protein microarray

7.2.1 Allergen

Horses are exposed to a wide variety of respirable particles in their everyday environment, including fungi, pollen, bacteria, as well as those of plant, animal and insect origin (Clarke and Madelin, 1987c; Woods et al., 1993). It is well documented that exposure to these respirable particles results in respiratory distress in sEA horses (Pirie, 2014b). There is however limited research on the precise contents of the respirable particulate fraction (Ivester et al., 2014). Ergo, allergen selection was largely limited to that previously identified as being present in the stabling environment (including hay and bedding) and those known to be in the horse's environment (e.g. pollens from common pasture/forage species) (Clarke and Madelin, 1987b; Couetil et al., 2015; Eder et al., 2000a; Seguin et al., 2010; Séguin et al., 2012; Terho et al., 1982). The microarray is limited by the fact that the identification of specific IgE sensitisations will only occur when the causative allergens are included on the array, however the plethora assessed in this study (n=384) significantly builds on the current knowledge of sEA-associated allergen due to the limited panel (<25) previously assessed (Marti et al., 2015).

As so few aeroallergens in the horse's environment have been accurately established down to the specific strain (Clarke and Madelin, 1987c), it was essential to utilise protein extracts to maximize coverage of the microarray at this initial stage, while maintaining specificity with the inclusion of pure proteins where the allergen were pre-established or readily available (Eder et al., 2001;

Künzle et al., 2007). Protein extracts are regularly utilised in type I hypersensitivity diagnostics, allowing evaluation of the patient's serum against all potentially allergenic particles of the source material. One concern with this is that the extract will contain both species specific proteins along with other common component proteins which will cross react with proteins from other origins, making true allergen sensitivity source identification difficult. Moreover, standardisation of allergen extracts is problematic as allergenic extracts produced by different suppliers have been shown to differ in their composition and activity, thus leading to differing results (Casset et al., 2013; Larenas-Linnemann et al., 2011; Wood et al., 2007). For these reasons, standardised recombinant allergen and highly purified components of allergen extracts enable more accurate and repeatable results. Although a larger number of recombinant proteins have become available in recent years, the numbers are still limited and do not cover the full range of potential allergenic proteins contained in a protein extract. For this reason, individual allergenic components are not generally recommended for the initial identification of an allergen source as can give a false-negative result. Therefore, allergen diagnostics are generally carried out with whole extracts, whereas molecular-based allergen diagnostics are used to establish the individual allergenic components in polysensitised individuals to identify the causal allergenic components, thus enabling allergen specific immunotherapy and further diagnostic/therapeutic advancements (Marth et al., 2014). Some authors have suggested analysis with recombinant allergens increases diagnostic accuracy (Caballero et al., 2012; Maruyama et al., 2016), whereas others suggested the use of the recombinant component alone may be insufficient for some

allergens (Smoldovskaya et al., 2016). The microarray platform gives us a rare opportunity in which we can simultaneously test extracts and pure/recombinant proteins using a small amount of blood (paper I and II). In paper II, we obtained similar accuracy utilising natural extracts (Hev b) and recombinant proteins (Hev b 3.0101; Hev b 5.0101; Hev b 6.02; Hev b 11). Although the eventual goal will be to move towards component resolved diagnostics (CRD) utilising individual allergen molecules for increased sensitivity and minimising cross-reactivity, the genus/species must be identified to enable the production of pure proteins.

Bacterial protein extracts are not readily available from commercial suppliers, as these are mainly focused on mite, pollen, mould and food allergens. Similarly, the strains of fungi identified as being of interest were not always commercially available, and as such it was necessary to produce these in-house. Strains were grown in liquid media, according to the suppliers recommendations or previously published literature, to enable effective removal of growth media and washing (Park et al., 2016). Liquid media is commonly utilised for the propagation of a large number of organisms. This may however have limited the analysis of several fungi extracts, as the hyphae of filamentous fungi aggregate to form pellets when grown under liquid culture conditions, this can prevent effective sporulation (Miyazawa et al., 2016). The sonication technique is the most popular technique for small scale cell lysis, using high intensity sound waves to disrupt cells (Morán et al., 2011). Samples underwent sonication of 4 x 30 seconds on ice, as previously described (Jouany et al., 2009). Careful consideration was given to this protocol as

temperature, time, and power setting can all influence total protein extraction and size distribution (Morel et al., 2000). The BCA (Bicinchoninic Acid) assay was utilised to quantify protein quantity and normalise samples to 0.5 mg/ml as previously described (Renault et al., 2011). The BCA assay is considered the “gold standard” over other protein quantification methods, such as NanoDrop (Noble et al., 2007; Singh et al., 1992).

The effect of freezing aqueous allergenic solutions is well reported (Vijay et al., 1987), and when frozen often results in damage to the protein structure through the formation of ice crystals. Therefore the addition of a cryopreservative in the form of glycerol, as previously utilised, was replicated (Wulfert et al., 2012).

7.2.2 Microarray printing

Initial microarray printing (Paper I) was conducted using a QArraylite arrayer (Genetix, UK), this was a single solid pin contact printer as previously described (Renault et al., 2011), meaning it functions through direct contact with the Nitrocellulose slide, transferring the allergen sample from a 384-well plate. This technique was largely popularised by its initial application in DNA microarray printing and later applied to protein microarray printing. Although well utilised in published literature, this contact technique can have several limitations, resulting in inaccuracy and inconsistency due to samples altering liquid, surface and colloidal properties (Cho and Bright, 2002). Firstly, the sample volume picked up by the pin depends on the surface tension and

adhesion forces to overcome gravity. The print pin's material and surface finish, cleaning rinse routine, and chemical composition of the print solution all play major roles in their capillarity and therefore the final print quality. Each time the pin strikes the surface a small change can occur in spot size and deposition volume. Split pins were not used as all allergen preparations were stored with glycerol as a cryopreseritive, making the solution viscous; causing issues with clogging and cross over. Solid pins are often utilised to combat this, preventing issues with cross contamination which would otherwise be a risk factor with viscous printing with pins containing a lumen or revivor. Conversely, solid pins are quickly and effectively cleaned between spotting different allergens with the use of a simple wash reservoir. This washing between each sample does however increase the time of printing, and increase the risk of fluidic drying, while also increasing print cost and complexity. As is common, the solid pin printing used in paper I utilised an environmental chamber, which encloses the microarray spotter controlling humidity, ambient dust and array substrate contamination. Moreover, this aids in the prevention of substrate and reagent evaporation. Influence droplet drying can be an issue with solid pins as pin evaporation can be problematic in terms of final volume and concentration. Moreover, this can result in loss of protein functionality, due to destabilisation of the secondary and tertiary structures. Therefore, controlling environmental conditions is essential for repeatable spotting and reliable analysis, as such paper I used humidity and environmental controls as previously described by Renault et al., (2011).

For these reasons, the final microarray slides (paper II) were professionally printed by Arrayjet (Roslin, Scotland) using an Ultra Marathon II by Arrayjet (Roslin, Scotland) which uses inkjet printer technology with piezoelectric nozzles to provide non-contact printing. Using this technique, as described in Marti et al., (2015) no contact is made between the printing tool and the nitrocellulose surface of the microarray slide, which is particularly desirable as contact can be detrimental to nitrocellulose. The technique is based on inkjet printer technology and uses piezo actuation to eject the protein solutions which are stored in the printer's ink cartridge. This is a result of a volumetric change in the ink reservoir which induces an abrupt pressure change in the reservoir resulting in droplet ejaculation. Using this technique, the piezoelectric nozzle precisely controls the drop volume, which can easily deposit multiple spots simultaneously, prevent contact breakages with the nitrocellulose and provides excellent position accuracy (Boland et al., 2006). Moreover, piezoelectric technology prevents sample heating or altering during printing. Accumulation of protein solutions within the ink head have been reported as the primary challenge of this technique, however, optimisation of humidity, surface blocking, protein stabilisers etc. circumvents this.

7.2.3 Anti-horse IgE

During optimisation in paper I, the monoclonal mouse anti-horse IgE 3H10 used was that originally produced by Wilson et al., (2006). As it is unrealistic to expect further advancements of this technique based on an antibody that is not readily available, I trialed the original Wilson et al. (2006) 3H10 clone with

the commercially available BioRad 3H10 (MCA5982GA). The BioRad 3H10 clone was chosen as it is the only one of this particular clone commercially available and has been utilised for a similar application previously (Einhorne et al., 2018). As monoclonal antibodies originate from hybridoma cells which secrete specific monoclonal antibodies to a single epitope, lot-to-lot variation is generally low, however this needs to be established when selecting a new source. Unpublished work by Wagner, Wilson and Marti demonstrated through comparability work that anti-IgE mAbs, even of varying production methods react similarly during ELISA and immunohistochemistry. This work demonstrated the excellent correlation between the Wilson et al. (2006) and BioRad 3H10 clones and thus identified an appropriate and replicable alternative.

7.3 Hay analysis

7.3.1 Hay collection

Post-collection, bales underwent treatment (see 8.3.2) and sampling (paper III). Hay was collected from each corresponding yard. Surveys were undertaken at the time of sample collection (pertaining to all Belgium samples) confirming all horses had consumed hay for a minimum of 3 months prior to collection. Originally, the French samples described in Orard et al., (2018) (paper II) underwent simultaneous hay treatment and collection to enable analysis of hay samples. Unfortunately, due to the nature of the collection

protocol utilised by Orard et al., (2018), the horses may not have become sensitised to the constituents contained in the hay, as although the horses had access to the hay for one month prior to commencing the study, horses were housed outside with the intention of avoiding respiratory crisis. As such, when originally assessed, sensitisation to many constituents was not seen. Therefore, the decision was made to use the mixed environment Belgium samples, which had certain prolonged enclosed exposure to the particulate fraction of the hay they were fed.

Hay was collected from each corresponding barn and underwent treatment (8.3.2) prior to sampling. As hay underwent treatment prior to sampling, it was not possible to use a coring devise. Coring devices are considered the "gold standard" technique for hay sampling as enables representative sampling throughout the entire region of the bale. As the hay was opened from the bale to undergo treatment, and due to wetting treatments, grab sampling was the only viable option. Twenty "grab" samples (each yielding approximately 25g) were taken at random across the sampling area in a W-formation. Literature advises a minimum of twenty samples to enable accurate representation of the sampled hay (Putnam, 2002).

7.3.2 Hay treatment

Hay treatments (paper III) were conducted essentially as previously described in Moore-Colyer et al., (2014), with the only variation being the steam treatment. In Moore-Colyer et al. (2014) hay was steamed in the HAYGAIN

600 for 40 minutes, to ensure it reached the manufacturer's recommended internal bale temperature. In Paper III hay was steamed in the HAYGAIN 600 hay steamer, once the integral thermometer reached 80°C (reflective of an internal hay temperature of 100°C according to the manufacturer) the hay was steamed for 10 minutes, and Digi-Sense Irreversible High Temperature Labels used to confirm an internal temperature of 100°C (Propress Equine Ltd, Hungerford, UK). Varying hay soaking methods have been used in the literature with soaking times from 0 min (Blackman and Moore-Colyer, 1998) to 16 hours (Clements and Pirie, 2007b), and water temperatures ranging from 8°C (Longland et al., 2011) to 39°C (Martinson et al., 2012), however all methods include full emersion of the hay. The most commonly used soak time and temperature in the literature was reported at 16°C for 10 min, *ergo* this was the final protocol utilised in paper III (Moore-Colyer et al., 2015, 2014).

7.3.3 Hay protein extraction

Previous hay analysis has utilised a Lab Blender 80 with peptone saline solution (Moore-Colyer et al., 2014), however, although this method may be appropriate for microbial assessment of forage, it was deemed insufficient for the analysis of allergenic proteins. Therefore, we utilised an optimised version of the Renault et al. (2011) food extraction protocol. This ensured full protein extraction, while preventing any overheating. Cell lysis disturbs the cellular environment and can result in unregulated endogenous proteases. As such,

protease inhibitor was included in the extraction buffer to prevent the extracted proteins from becoming degraded or modified.

7.3.4 Hay microarray protocol

The competition microarray was largely based on competition/inhibition techniques outlined in Lin et al., (2009) and Renault et al., (2011) in order to assess the quantities of specific allergen present in an environment for a specific patient. Serum was pre-incubated in a competitive hybridisation for 1 hour at room temperature (Lin et al., 2009; Renault et al., 2011) with 1/10 of the hay protein extract (Renault et al., 2011).

7.4 Statistical analysis

When performing optimisation techniques and hay analysis (paper I and III) linear regression was used to assess correlation, and analysis of variance (ANOVA) with Tukey's post hoc used to establish differences between groups. Z-test was used to assess differences between the control and sEA-affected groups during the optimisation process (paper I). Z-tests have previously been used to analyse microarray fluorescent units (Cheadle et al., 2003), takes into account distribution across the population, and is ideal for significance estimates offering a useful method for the basic analysis of microarray data. As each protein is assessed individually, this means a large number of statistical tests are made simultaneously. With this type of multiple testing it is important to apply corrections when assessing differences in sIgE levels to

prevent false positives. For example, 384 proteins/extracts (paper I) evaluated with a p-value of 0.05 to determine proteins associated with sEA, then at 5% there would be 19 positive findings merely by random chance, to account for the potential number of false positives, the p-value must be corrected. Certain methods for this such as the Bonferroni correction, which controls the familywise error rate (FWER), can be too constrictive, potentially resulting in true discoveries being discarded. *Ergo*, the Benjamini–Hochberg False Discovery Rate (FDR) approach was used to be less constrictive.

For classification methods (paper II) a predictive mathematical model was created as previously described in Marti et al. (2015). In this procedure, Partial Least Squares Discriminant Analysis (PLS-DA) was utilised to separate pre-defined classes, by effectively performing partial least squares regression against a dummy matrix. By doing this, the model is able to identify the specific allergens (variables) which are most significant for class prediction (Lee et al., 2018). Each variable is assigned a variable influence on the projection (VIP) score as a weighted sum of the squared correlations between the original variable and the PLS-DA components. This is a measure of the contribution that a specific variable has on the model (Adamko et al., 2015). In order to test the mathematical model produced, multiple rounds of cross validation (CV) were performed using different partitions, and the validation results were amalgamated through the rounds giving an estimate of the model's predictive performance (Adamko et al., 2015).

7.5 References

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8.0 General discussion

Little is known about possible allergen sources for sEA horses, and particularly the responsible allergen molecules. In paper I and II it was established that specific IgE can be measured using the specially designed protein microarrays developed in the first stages of this study, and could be used as a biomarker to establish allergens recognised by sEA-affected horses (Couetil et al., 2015; Eder et al., 2000c; Halliwell et al., 1993a; Künzle et al., 2007; Niedzwiedz et al., 2015; Schmallenbach et al., 1998). This type of microarray, miniaturised allergy diagnostics, was first utilised by Hiller et al. (2002), for sIgE profiling of potential respiratory allergen in humans. Later microarray allergology experiments proved to be comparable with well-established methods, such as ELISA, RAST and immunoblot. Several studies have utilised microarrays for sIgE profiling in humans, however this platform must be optimised and validated to enable its use in the veterinary diagnostics sector. Most protein microarrays consist largely of two steps: 1) protein printing onto the microarray slide, 2) immunolabeling of equine sera. Ergo, in paper I, these steps were optimised. Firstly, incubation conditions, biological fluid source, specificity, reproducibility between print lots, as well as, differing antibodies for immunolabeling were optimised prior to printing. Secondly, specificity was confirmed through a protein inhibition assay, demonstrating some non-specific inhibition. This optimised method, is of great value to the veterinary sector, advancing understanding of disease aetiology and pathophysiology, while enabling diagnostic and therapeutic advancement.

8.1 IgE in severe equine asthma

8.1.1 Specific IgE as a biomarker in severe equine asthma

The development of sIgE diagnostics for sEA has been complex to date. Initially, Eder et al., (2000) concluded that further work was required to establish the effects of environment, age and gender on sIgE and to investigate the role of IgE in the pathogenesis of sEA. Since then, several studies have evaluated the influence of mixed environments (Eder et al., 2000), age (Kunzle et al., 2007) and gender (Wilkolek et al., 2014), indicating variations between environmental conditions, but minimal influence relating to age and gender. Moreover, a growing body of evidence has implicated the involvement of IgE in the pathogenesis of sEA (Moran et al., 2010 a,b); Kunzle et al., 2007; Curik et al., 2003; Eder et al., 2000; Halliwell et al., 1993; Schmallenbach et al., 1998). Despite increased understanding of the involvement and variations of sIgE, little advancement has been made in sEA sIgE diagnostics, predominantly due to the complexities of large-scale profiling using common techniques such as ELISA. Furthermore, previous sEA sIgE diagnostics were hampered by the limited availability of recombinant proteins, which tended to enable clearer class separation, than protein extracts (Eder et al., 2000). In subsequent ELISA work, Kunzle et al. (2007) concluded that although in controlled environmental conditions serological sIgE against mould was higher in sEA horses, the overlap between diseased and non-affected animals was too substantial to make the test of any diagnostic value. These difficulties arise from the use of statistical tests, such as the Mann-Whitney *U*-test to assess differences between raw fluorescent units in sEA and non-

affected horses, instead of binary classification models. Another limitation of sEA sIgE diagnostic work is the restricted number of horses included for analysis. In comprehensive and complex microarrays, because of the large quantity of variables it is best to avoid direct regression analysis as this will result in spurious results and poor convergence. These notorious issues were avoided here by using a multiparametric approach. To establish the relevance of differing variables in sEA discrimination against the various other clinical groups, a PLS-DA was utilised for classification analysis. Paper II demonstrated the strength of sIgE as a biomarker for sEA diagnostics by applying binary classification methods commonly utilised in human work (Fiocchi et al., 2010). Binary classification models, such as, partial least squares discriminant analysis utilised in paper II have been hugely successful in the bioinformatics field within human medicine (Adamko et al., 2015). PLS-DA models have been used to demonstrate the accuracy of asthma classification via urine metabolomics profiling (Saude et al., 2011). Using these binary classification techniques, as oppose to the standard comparisons between fluorescent units previously utilised in equine work, it enabled clear classification (paper II) where others have struggled (Kunzle et al., 2007). Moreover, such disease classification methods identify allergens associated with the aetiology of sEA. Despite the multitude of benefits to such approaches, the use of diagnostic mathematical models is currently not utilised by worldwide clinical laboratories due to the quantity of data required to build a reliable model, and the complexities in producing user-friendly interfaces for such systems.

It is well acknowledged that pure/recombinant proteins achieve a greater standardised allergen preparation in contrast to protein extracts (Valenta et al., 2018). Therefore, pure/recombinant proteins are increasingly utilised to increase specificity in allergology (Curin et al., 2017). In humans, component resolved diagnostics derived specific immunotherapy has a much higher success rate (Canonica et al., 2013), which would suggest that the future of equine diagnostics should also be based on microarrays utilising pure/recombinant proteins. As discussed in previous papers (Renault et al., 2011; Marti et al., 2015), the numbers of potential allergen are immense. For example, several important domestic crops in the plant kingdom actually have genomes that are several times bigger than the human genome. *Ergo,* even when accounting for different polymorphisms and their polyploidy nature, it would be impossible for a singular microarray to cover all proteins. For this reason, a hybrid version incorporating pure/recombinant proteins and extracts (as presented in paper I, II and III) is considered the best that can be achieved at present. This is supported by data to date, which suggested a combination of extracts and pure proteins in an ‘aggregated response’ would be best practice. In terms of immunotherapy, particularly for sEA horses, it must be acknowledged that although a good understanding of potentially inciting allergen has been established (e.g. r Asp f 8), future microarrays should include a wider panel of environmental proteins.

This work also has wider implications, as comparative allergology between humans and their domesticated pets is key to the World Health Organisation’s “One Health” concept incorporating animal, human and environmental health (Einhorne et al., 2018).

Diagnosis of allergy conditions and immunotherapy is commonly conducted using protein extracts in domesticated animals (Greer et al., 2019). Similarly, intradermal studies utilising protein extracts is common practice in equine allergy diagnostics, although comparative accuracy of this with conventional serological tests has been controversial (Tahon et al., 2014). This emphasises the requirement for a sensitive serological method for sIgE profiling, where blood can be quickly and easily collected on site without the requirement for sedation and the more invasive BALF collection. Antigen microarrays should be utilised as a novel diagnostic approach for horses with suspected allergies/asthma, identifying sensitisation profiles that enable the development of therapeutic, diagnostic and avoidance strategies.

8.1.2 Allergens previously implicated in the aetiology of sEA

SIgE analysis has previously been utilised to identify the predominant allergens associated with sEA and in human asthma. The results from paper I and II, where the large number of sEA allergens identified included those previously identified by sIgE methods i.e. *Wallemia sebi, Geotrichum candidum, Eurotium amstelodami, Aspergillus terreus, Saccharopolyspora rectivirgula, Tyrophagus putrescentiae, Alternaria alternate, rAsp f 8,* Asp f 1/a), and *Aspergillus fumigatus* (*Asp f* (extract))(Couetil et al., 2015; Eder et al., 2000c; Halliwell et al., 1993a; Künzle et al., 2007; Niedzwiedz et al., 2015;

Schmallenbach et al., 1998), demonstrates sIgE is a reliable method for sEA diagnosis.

8.1.2.1 Fungi

Several fungal allergens were found to be significant in diagnosing horses with sEA, these included *Eurotium amstelodami* (Eur a), *Geotrichum candidum* (Geo c), *Mucor circinelloides f. lusitanicus* (Muc ci) and *Aspergillus fumigatus* (rAsp f 8) (see table 1).

Table 1: Significant fungal sEA-associated allergen identified in sera (paper I and II), and potential fungal allergen identified in hay (paper III).

Paper I		**Paper II**		**Paper III**	
Aspergillus restrictus	Asp r 1	*Aspergillus fumigatus*	rAsp f 8	*Malassezia Pachydermatis*	Mala p
Penicillium notatum	Pen ch	*Mucor circinelloides*	Muc ci	*Aureobasidium Pullularia Pullus*	Aur p
Malassezia pachydermatis	Mala p	*Aspegillus flavus*	Asp fl	*Penicilium notatum*	Pen ch
Alternaria alternata	Alt a 1	*Geotrichum candidum*	Geo c	*Rhizopus nigrican/stolo*	Rhi n
Aspergillus fumigatus	Asp f	*Aspergillus versicolor*	Asp v	*Alternaria alternaria tem*	Alt a
		Penicillium expansum	Pen e	*Aspergilus niger*	Asp n
		Cladosporium herbarum	Cla h	*Aspergillus genuine marker*	Asp r 1
				Helminthosporium spondylocladium	Dre h
				Aspergillus nidulans	Asp nid
				Candida albicans	Cand a
				Cladosporium herbarum	Cla h
				Aspergillus versicolor	Asp v
				Aspergillus fumigatus	Asp f
				Geotrichum candidum	Geo c

Differences between allergen identification between paper I and II are expected due to the increased range of sEA-relevant proteins selected for the second array. Moreover, although not identified in paper I, the potential cross-

reactivity between *Penicillium* and *Aspergillus*, due to galactomannans with similar galactofuranosyl and immunogenic side chains, as well as *A. fumigatus* with *Cladiosporium, Candida, Alternaria, Trichophytin,* and *Epidermophyton*, demonstrates the commonality between allergens identified in paper I and II.

The most significant fungal protein for class prediction in paper II was rAsp f 8, a protein not assessed in paper I. These rAsp f 8 results are in agreement with Künzle et al. (2007) and Eder et al. (2000) who both identified substantially higher levels of IgE against this recombinant mould allergen in sEA-affected horses (Eder et al., 2000; Künzle et al., 2007). Tahon et al. (2009) also found sEA-affected horses tested positive to rAsp f 8 intradermally compared to non-affected horses (Tahon et al., 2009b). This could be a result of IgE autoimmunity, which is well described in humans that suffer with severe allergies. Recombinant Asp f 8 is an *A. fumigatus* derived ribosomal P2 protein. In humans with severe atopic disease autoimmune reactions against phylogenetically highly conserved intracellular proteins, such as ribosomal P2 protein, are notorious and thought to result from cross reactivity. Furthermore, humans suffering with severe allergic bronchopulmonary aspergillosis, show both a positive intradermal test and increased sIgE against ribosomal protein P2 from *A. fumigatus.* It is hypothesised that this autoreactivity could result from tissue damage and the subsequent release of autoantigens at the inflammatory site, as in normal circumstances it is unlikely that these cytoplasmic proteins would be accessible for antigen/antibody interaction.

Mucor circinelloides f. lusitanicus (Muc ci) results further confirm previous research demonstrating *Mucor* allergen extract sensitisation is associated with sEA-affected horses via *in vitro* basophil assay (Dirscherl et al., 1993). Similarly, increased levels of specific IgE against *E. amstelodami* and *G. candidum* have been identified in the bronchoalveolar lavage fluid of sEA affected horses via Western blot (Couetil et al., 2015).

Fungi has long been associated with sEA, as in human allergic asthma, the greatest potential aeroallergen exposure comes from fragmented hyphae as oppose to conidia, particularly in light of their ability to produce a plethora of diffusible allergens (Green et al., 2006). Moreover, tracheal washes in sEA horses isolated fungi belonging to the genera *Penicillium, Cladosporium, Alternaria* and *Aspergillus* (Xavier et al., 2014). Hay has long thought to be a predominate source of fungi to the horse and has previously been shown to induce sEA through inhalation trials. Further work has confirmed the potential sEA inciting allergen that may be present in hay (Seguine et al., 2015). For the first time, paper III utilised the developed microarray platform to identify the presence of specific fungal allergens present in hay (see table 1); this information is crucial in devising appropriate allergen avoidance strategies. Although hay treatments did not have an effect on IgE-protein binding of the allergen assessed, caution should be taken when soaking hay, as fungi can generally grow well in water, largely dependent on the quantities of dissolved solids. In this scenario, soaking hay provides a wide range of solids, such as cellulose and water-soluble carbohydrates. This could potentially lead to proliferation of fungi, which although may not be inhalable

due to the soaking, will lead to inoculation of the horse's environment and subsequent exposure.

8.1.2.2 Arthropods

Several arthropods were found to be significantly associated with sEA in both paper I and II (see table 2), including the proteases from *Blattella germanica* and *Dermatophagoides farina*, *Dermatophagoides pteronyssinus* and *Tyrophagus putrescentiae.*

Table 2: Significant arthropod sEA-associated allergen identified in sera (paper I and II), and potential arthropod allergens identified in hay (paper III)

Paper I		**Paper II**		**Paper III**	
Dermatophagoides farinae	Der f 2	*Blattella germanica*	Bla g 2	*Blattella germanica*	Bla g 2
Blattella germanica	Bla g 5	*Dermatophagoides farinae*	Der f 1	*Dermatophagoides farinae*	Der f 1
Dermatophagoides pteronyssinus	Der p 7	*Dermatophagoides pteronyssinus*	Der p 2	*Dermatophagoides pteronyssinus*	Der p 1
B germanica	Bla g 5	*Dermatophagoides pteronyssinus*	Der p	*Dermatophagoides pteronyssinus*	Der p 7
		Blatta orientalis	Bla o	*Lepidoglyphus destructor*	Lep d
		Dermatophagoides pteronyssinus	Der p 10	*Tyrophagus putrescentiae*	Tyr p
				Euroglyphus maynei	Eur m 2

House dust mites (HDM) are prevalent in stabling and general household environments and are implicated in the perpetuation and initiation of human and equine asthma. The HDM are regularly transported by feed materials (e.g. hay), bedding and domestic animals. The enzymatic potency from HDM is crucial for specific sensitisation, as well in initiating inflammation. Furthermore, HDM induce a Th 2 response and upregulation of TSLP, IL-33 and IL-25 through epithelial intelectin. Horses with sEA appeared to be sensitised to Der

p 2, a major human HSD allergen. Einhorne et al. (2018) found in the general population of horses, reaction was most common to papain, a Der p 1 structural and functional homologue with protease function (serine). Moreover, several commercially available shampoos contain papain which can result in percutaneous sensitisation. It should be noted that several minor mite allergens were not included in the microarray (e.g. hemocyanin / sarcoplasmic calcium-binding protein / arginine kinase), however have shared allergenic components with shrimp and insect allergies, in which case they can also potentially be primary sensitisers. This explains the sensitisation found in paper I and II to both shrimp tryptomycin and *Helix aspersa* Hel as 7, as well as Bla g 2, which may indicate cross-sensitisation to corresponding HDM allergens, or parasites currently not included on the array. Moreover, it must be considered that IgE binding of these proteins could potentially result from cross-reactivity, for example, from amylases and serine proteases present in *Tyrophagus putrescentia* as well as in other allergens, such as *Dermatophagoides* or *Blatella*.

Moreover, the array results therefore ratify recent reports of the involvement of *Acarus siro*, *Dermatophagoides, farinae/pteronyssinus, Tyrophagus putrescentia* in sEA and their association with high concentrations of specific IgE against mites, particularly *T putrescentia* (Niedzwiedz et al., 2015). Bla g 2 is associated with the development of asthma in humans and increased sIgE against Bla g and has previously been reported in sEA-affected horses (Pomès et al., 2002). Tropomyosin results (Hel as 1 and Per a 7) are to be expected, as Tropomyosins are major allergenic components accounting for cross-reactivity with mites and other arthropods (Xi and Sun, 2016).

Furthermore, the high VIP scores demonstrated for *Cullicoide* proteins (Cul nu 2, CO145, Cul o 2) could have resulted from the sEA/IBH horses, even though these were matched with IBH controls, or from multiple hypersensitivities, as sEA horses are at increased risk of IBH (Kehrli et al., 2015) which is associated with airway hyper-reactivity (Lanz et al., 2017).

Paper II demonstrated that a majority of these major arthropod allergen were detectable in hay (see table 2). Although the presence of mites has previously been assessed, this has generally been via microscopy analysis. The microarray technique utilised in paper III enabled accurate detection utilising recombinant proteins, this is particularly crucial considering the high levels of cross-reactivity reported among whole extract preparations of mites. Moreover, this method enabled the accurate confirmation of hay as an arthropod allergen source for those identified in paper I and II. Although hay treatment methods examined in paper III revealed no alteration in IgE-protein binding for arthropods, it is worth noting that proliferation of fungi in hay is associated with increases in corresponding mites.

8.1.2.3 Bacteria

Few bacteria strains are singularly implicated in the aetiology of sEA, with many authors suggesting the main causal bacterial antigens to be *Saccharopolyspora rectivirgula* and *Thermoactionmyces vulgaris* (Moran et al., 2010).

Table 3: Significant bacteria sEA-associated allergen identified in sera (paper I and II), and potential bacteria allergen identified in hay (paper III).

Paper I	Paper II		Paper III	
N/A	*Thermoactinomyces vulgaris*	The v	*Kineosporia rhizophila*	Kin r
	Kineosporia rhizophila	Kin r	*Aeromicrobium ponti*	Aer p
			Acinetobacter gerneri	Aci g
			Erwinia rhapontici	Erw r
			Thermoactinomyces vulgaris	The v

The most significant sEA-associated bacteria from paper II was *Thermoactinomyces vulgaris*, which has long been associated with sEA and increased levels of IgE in affected horses (McGorum et al., 1993b; Morán et al., 2010; Pirie, 2014b). The lack of bacterial identification from paper I is largely explained by the decreased panel of bacterial proteins assessed at this optimisation stage. Bacterial analysis in paper III identified several strains associated with the equine environment and/or previously implicated in sEA, including *Kineosporia rhizophila*, *Thermoactinomyces vulgaris*, and *Acinetobacter gerneri*.

The integrity and hydration of bacteria fungi cells is largely dictated by the osmotic pressure of their environment, known as hydrostatic pressure. It is possible that the sudden increase in water influx into the cell causes swelling and even lysis, thus rendering the cell inactive (Wood, 2015). However, many “wild” bacteria have mechanisms that can protect against this and ensure survival despite excessive water exposure, as have selectively survived osmotic stresses in the first place. Similarly, it has been reported that although analysis of the post-soak liquor would indicate that some bacteria were washed off during the soaking process, others were able to survive and proliferate in the water; resulting in a 1.5-fold increase in hay bacterial counts after just 10 minutes soaking (Moore-Colyer et al., 2014). To date, the identity

of the bacteria which proliferate, and the impact this may have on the animal has not been identified. Conversely, when the individual sEA-associated bacteria were assessed (paper III), there was no increase in the level of specific bacteria present in soaked hay. Further work utilising 16s rRNA sequencing in conjunction with propidium-monoazide (which enables the accurate assessment of live bacteria) is required to establish the effect that soaking has on the proliferation of specific bacterial strains, as these results indicate. Another valid consideration is the effect that the water source may have on microbial proliferation. Unlike many other countries, the UK has low quantities of chlorine present in drinking water, and therefore may be more favourable to microbial proliferation when compared with the high chlorine levels of countries such as USA (LeChevallier et al., 1991). Moreover, this work demonstrates that even if lysis occurs due to osmosis, the protein is still present and able to be recognised by sIgE.

8.1.2.4 Pollen

In general, pollen results were representative of the known susceptibilities in the horse population to grass and tree pollens (see table 4). Paper I had a limited range of pollens included compared with paper II resulting in limited identification.

Table 4: Significant pollen sEA-associated allergen identified in sera (paper I and II), and potential pollen allergens identified in hay (paper III)

Paper I		Paper II		Paper III	
Linum usitatissimum	Lin us	*Betula verrucosa*	Bet v 2.0101	*Annual Mercury Profilin*	Mer a 1
Triticum polonicum	Tri tp	*Mercurialis annua*	Mera1	*Arroyo Willow*	Sal la
Actinidia chinensis	Act c 5	*Prunus Persia*	Peru p 3	*Barley Pollen*	Hour v
Actinidia deliciosa	Act d 11	*Quercus robur*	Que r	*Blood Amaranth*	Amal cry
Olea europaea	Ole e 2	*Actinidia deliciosa*	Act d 2	*Blue Mussel*	Meet e
Anthoxanthum odoratum	Ant o	*Parietaria judaica*	Par j 1	*Chestnut Pollen*	Cas s
Parietaria judaica	Par j 1	*Corylus americana*	Cor am	*Corn Pollen*	Zea m
Triticum turgidum ssp. *durum*	Tri td	*Lupinus albus*	Lup a	*Cypress genuine marker*	Cup a 1
		Zeal mays	Zeal m	*Durum Wheat*	Tri td
		Olea europaea	Ole e 1	*Fennel dog Eupatorium ca*	Eup c
		Olea europaea	Ole e	*Fescue meadow, Festuca pratense*	Fes p
		Arachis hypogaea	Ara h 6	*Hazel pollen Polygalacturonase*	Cor a PG
		Gallus domesticus	Gal d 4	*Hazelnut Europenan orylus avellan*	Cor a
		Parietaria judaica	Par j 1	*Sorghum halepense*	Sor h
		Helianthus annuus	Hel a	*Poa pratensis*	Poa p
		Corylus avellana	Cor a	*Chenopodium al*	Che a
		Olea europaea	Ole e 2	*Chrysanthemum leu*	Leu vu
		Fagopyrum esculentum	Fag e	*Oak pollen*	Que i
		Eucalyptus globulus	Euc g	*Olea europaea*	Ole e 1
		Quercus ilex	Que i	*Dactylis Glomerum*	Dac g
		Actinidia chinensis	Act c 10	*Palm Profilin*	Pho d 2
		Artemisia vulgaris	Act v 1	*Parietaria LTP*	Par j 1
		Ricinus communis	Ric c	*Ragweed genuine marker*	Amb a 1
		Avena sativa	Ave s	*Agrostis gigantus*	Agr g
		Periplaneta americana	Per a 7	*Lolium Perenne*	Lol p 1
		Ceratonia siliqua	Cer si	*Lolium Perenne*	Lol p
		Castanea sativa	Cas s	*Sunflower Pollen*	Hel a
		Armoracia rusticana	Arm r	*Anthoxanthum*	Ant o
		Taraxacum officinale	Tar o	*Sycamore Genuine marker*	Pla a 2
		Platanus acerifolia	Plaa8	*Phleum pratenses*	Phl p

	Triticum aestivum	Tri a	*Phleum pratenses*	Phl p 7.0101
	Carpinus betulus	Car b	*Holcus lanatus*	Hol l
	Leucanthemum vulgare	Leu vu	*Wheat Gliadin fraction*	Tri a Gliadin
	Brassica	Bra n	*Wheat Pollen Tri a*	Tri a
	Acer saccharinum	Ace s		

Interestingly, the main sEA-associated pollen identified in paper II, pollen allergen Bet v 2.0101 from birch pollen, is from the botanic species *Fagales* which in turn belongs to a family of proteins responsible for plant innate immune function, known as PR-10. These PR-10s can elicit a Th 2 immune response due to their ligand binding ability, in both animals and humans. Moreover, they can sensitise human atopic individuals and are highly cross-reactive. This is usually driven by a strong IgE response to Bet v 1 in the human (Hauser et al., 2011). Einhorne et al. (2018) identified the allergen alder pollen Aln g 1, another PR-10, as a major respiratory sensitiser. However, this molecule was not included in the developed microarray platform utilised in paper I, II and III. Einhorne et al. speculated that the predominant tree species present around the paddock may represent the primary sensitising allergen source. This theory is however conflicted with reports of human Bet v 1 sensitisation in birch-free regions. Interestingly, paper II showed 28 pollens were significant for class separation, including the aforementioned *Betula verrucosa* (Bet v 2.0101), *Mercurialis annua* (Mer a 1), *Eupatorium capillifolium* (Eup c), *Quercus robur* (Que r) and *Helianthus annuus* (Hel a). The Bermuda grass pollen Cyn d has previously been identified as a common source of sensitisation in horses, but Cyn d and Cyn d 12 were not significant in class prediction (Einhorne et al., 2018). To the

author's knowledge, this is the first study to show an association between sEA in horses and a hypersensitivity to pollens.

Historically, pollen exposure has been considered to be 'location dependent' and a result of external (i.e. pasture) environmental exposure, as seen in human hay fever. Paper III has demonstrated that conserved forages are a prolific source of pollen. This may have huge implications for, not only the management of sEA, but also summer pasture-associated sEA, which is more associated with pollens. With this condition, owners often avoid turnout at peak pollen times, however avoidance of respirable pollens in conserved forages may also be an important consideration, particularly in-light of the high cross-reactivity of sensitising grass pollens.

8.1.2.5 Latex and others

The most significant genus associated with sEA in paper I and more notably II, was from natural rubber latex (Hevea brasiliensis, Hev b), these included Hev b-extract; Hev b 3.0101; Hev b 5.0101; Hev b 6.02 and Hev b 11 (see table 5). This is the first time Hev b allergens have been assessed in relation to sEA.

Table 5: Significant Hev b sEA-associated allergen identified in sera (paper I and II), and potential Hev b allergens identified in hay (paper III)

Paper I		Paper II		Paper III
Hevea brasiliensis	Hev b 11	*Hevea brasiliensis*	Hev b 11	N/A
Hevea brasiliensis	Hev b 5.0101	*Hevea brasiliensis*	Hev b 6.02	
Hevea brasiliensis	Hev b 6.02	*Hevea brasiliensis*	Hev b 5.0101	
		Hevea brasiliensis	Hev b 3.0101	
		Hevea brasiliensis	Hev b	
		Hevea brasiliensis	Hev b 9	
		Hevea brasiliensis	Hev b 8	
		Hevea brasiliensis	Hev b 1	

The top 3 Hev b allergen associated with sEA between paper I and II were in agreement. Work in human asthma patients has revealed a higher frequency of Hev b allergies in affected people (Uspenskaia, Luss, & Babakhin, 2011). This was also shown to be true for horses in paper II, where IBH horses did not show an increased sensitisation to Hev b molecules. This is particularly important, as it has previously been suggested that a human/animal prone to allergies can result in concurrent allergies and/or sensitisations. The fact that increased Hev b sensitisation is seen in sEA horses and not IBH horses confirms the specificity of the disease.

Exposure to latex can occur through the inhalation of airborne particles, blood stream, mucus membranes, and direct contact (skin). A major source of respirable Hev b allergens in the horse's environment is from artificial riding surfaces. Many parts of Europe have banned the use of recycled tyres, however, in the UK it is permitted under current Environment Agency waste regulations (Waste Exemption: U8 use of waste for a specified purpose). Also, throughout the world many arena surfaces contain components of natural

rubber including those where the samples were collected in paper I, II and III. These artificial surfaces have previously been associated with chronic bronchitis in riding instructors due to high levels of respirable dust. (Claußen & Hessel, 2017; Kollar, Swinker, Swinker, & Irlbeck, 2005; Lühe, Mielenz, Schulz, Dreyer-Rendelsmann, & Kemper, 2017). In the lungs of humans it has been shown that inhalation of Hev b particles induces inflammation. (Donaldson et al., 2000). Furthermore, particles, such as Hev b, have been shown to exhibit an adjuvant effect by increasing the primary response during sensitisation when present either before, during or after allergen exposure (Granum et al., 2001). Diaz-Sanchez et al. (1999) demonstrated particulate inhalation during allergen exposure could induce a mucosal IgE response under conditions in which the allergen alone could not (Diaz-Sanchez et al., 1999).

In humans, it has been demonstrated that latex proteins fix to powder used in the manufacturing of latex gloves. When the glove is used, the powder/latex particulate become airborne and easily inhaled, as well as entering through the mucus membranes. Moreover, latex-induced occupational asthma can result from repeated work place exposure to latex molecules. In occupationally-unexposed groups, the prevalence of latex sensitisation is substantially lower (<1%) than those regularly exposed to latex gloves (>18%)(Vandenplas et al., 2002). Interestingly, the main allergen associated with occupational latex-allergy (Hev b 6.02) (Yagami et al., 2009) was also the second most influential VIP in this study with sEA-affected horses, along with

other major Hev b-allergens used for occupational latex-allergy diagnosis (Hev b 5.0101; Hev b 11;)(Raulf et al., 2018).

Human latex-induced asthma and sEA both occur in genetically predisposed patients (Brown et al., 2005; Gerber et al., 2015). These results may suggest that this genetic predisposition associated with sEA may also predispose the horse to latex hypersensitivity, or that sEA may be driven by latex-induced asthma and subsequent particle exposure results in increased sensitisation to respirable environmental allergens the horse is regularly in contact with (i.e. in the stabling environment). Further work in relation to horses is required to establish the *in vivo* effect of latex particles in the aetiology of sEA and allergen sensitisation.

In humans, contact allergies are also reported with latex, these do not however result in asthma like symptoms, but rather itching, dryness, erythema, bleeding, and/or scaling. Although poorly researched, there are reports of contact hypersensitivity in horses, which similarly results in lesions to the area where contact occurs (Fadok, 1995). Substances reported to cause contact allergy include plants, bedding, shampoos, blankets, fly sprays and topical antibiotics such as neomycin. Stable matting, which often contains natural rubber latex, may have the potential to cause contact hypersensitivities, but is unlikely to cause asthma like symptoms.

The results from paper I and II have shown that latex is associated with sEA. Although further research is required, to evaluate latex exposure and confirm

the *in vivo* effects, these studies have highlighted an important, and often overlooked potential source of hypersensitivity. This will have a drastic impact on the management of all sEA affected horses, and the wider population, improving health, welfare and performance.

8.1.3 Effect of mixed environment

Differences between the matched and mixed groups (paper II) allergen-specific serum IgE levels might have resulted from different horse populations and environmental factors (e.g. exposure to mouldy hay), resulting in lower sensitivity and specificity values in the mixed group compared with the homogenous matched group. The fact that IgE levels in the horse are influenced by the environment is not surprising in light of human research in dizygotic and monozygotic twins that has demonstrated the substantial influence environmental factors play in specific serum IgE levels, on top of the genetic factors (Hopp *et al.* 1984). Here, paper II has demonstrated that environmental factors play an important role in relation to serum IgE levels and must be considered in subsequent studies, the sensitivity and specificity values from the mixed environment still proved to be effective for accurate diagnosis using the model. Moreover, many similarities were apparent between the matched (MA) and mixed group (MI), such as Der f and tropomyosin Pen i 1 in MA compared with Der f 1 and tropomyosin Hel as 7 in MA. The MI group was equally reliant on a range of *aspergillus* species (Asp v, Asp n, rAsp f 8), whereas the MA group primarily relied on rAsp f 8. Both groups were heavily reliant on Hev b molecules. Bovine milk proteins are important for class prediction in both models (MA - Bos d 4, Bos d 9; MI - Bos

d LF), the significance of this warrants further research. These molecules commonly cross react between species and have shared common allergenic components with other allergens, such as Glycine max. Further identification of common allergens, with specific focus on pure/recombinant proteins, would enable increased diagnostic accuracy within a mixed group. Further regional specificity in terms of allergens may also be required, such as the use of tropical grass pollens for certain regions in the United States of America. Exposure studies, and a global mixed sEA collaboration group would be necessary for further validation of this diagnostic technique.

8.1.4 Multiple Hypersensitivities

Clinical, epidemiological and genetic evidence all suggest that differing hypersensitivities can occur concurrently in horses, as in other species. Verdon et al. (2019) demonstrated horses concurrently affected with sEA and IBH did not have raised levels of sIgE to a select panel of recombinant proteins, when compared with singularly affected IBH or sEA horses. The findings of the developed array were in agreement with this. Verdon et al. (2019) found that sEA horses were not sensitised to recombinant *culicoides* allergens. Interestingly, the reason this study found certain cullciodes to be significant resulted from the potential bias grouping of sEA and sEA/IBH horses in the PLS-DA model. This resulted in the increased significance of IBH associated allergen, most notably r Cul o 1P, which was found to be significantly higher in the IBH group assessed by Verdon et al. (2019). In paper

II, a significant difference was identified between sEA and control using r Asp f 8 as reported in several previous studies. Interestingly, Verdon et al. (2019) were unable to detect any significant differences between the sEA, IBH and control groups using recombinant Asp f 7 and 8. The limited range of potential allergens tested at a single time has been a major limitation of multiple hypersensitivity work, the application of this developed microarray would be ideal to enable the assessment of several hundred potential allergens simultaneously.

8.2 Environmental testing via competition microarray

Environmental testing is seldom conducted in relation to the horse. Microbial assessment of forage and bedding samples has been conducted utilising pre-packaged selective media to identify quantities of active bacteria and fungi (Moore-Colyer et al., 2016), as well as further work utilising selective media to identify the specific strain present (Bauters and Nelis et al., 2000). The major limitation with this is that non-active bacteria and fungi are able to elicit an allergic reaction, so the quantities of these present and their subsequent ability to elicit immune responses are of interest in relation to sEA affected-horses. Classic methods, such as ELISA, may be used to identify specific pre-determined protein in an environment, but the protein of interest must be known. These methods are laborious, expensive and prevent wide-scale routine protein assessment of environmental samples. Moreover, in allergies mediated, or suspected to be mediated by IgE, the interaction between the immunoglobulin and specific protein is essential. As allergen avoidance is the

cornerstone of effective management and treatment of sEA, the lack of relevant wide-scale environmental assessment has posed issues for effective allergen-avoidance regimes.

The protein inhibition studies utilised in paper I, demonstrated the ability to accurately identify specific proteins using an adapted competition method. The novel application of this technique to assess a protein extract created from a given sample (e.g. hay), enabled identification of the specific IgE-protein binding in a given sample, as well as, a specific sensitisation profile for the individual animal. This offered a unique assessment of IgE-protein interactions, as well as, enabling the assessment of 384 potential allergen from a single test. This allowed for the identification of patient specific allergen sources. In paper III it was demonstrated that it was possible to identify over 40 potential allergens in one specific horse's environment, although this is highly dependent to the specific horse's sensitisation profile and array protein inclusion. This test enabled the widest-scale assessment of IgE-protein binding of a specific sample/patient combination on a microscale to date. It has huge application in the management of asthmatic horses and the development of tailored allergen avoidance regimes.

8.3 Hay for severe equine asthma affected horses

Using the aforementioned competition microarray technique, paper III demonstrated that differing hay preparation techniques, including soaking and steaming, did not alter IgE-protein binding. Historically, owners of sEA-

affected horses have predominately relied on soaking hay to reduce airborne respirable particulates, which is highly effective in this regard. The influence soaking may have on bacteria and mould is uncertain. It is well established that steaming hay in a HAYGAIN 600, as performed in paper III, reduced viable bacteria (99%), mould (99%) and respirable particle count (94%), indicating it is beneficial in reducing allergen exposure by decreasing respirable particulate matter (Moore-Colyer et al*.,* 2015; Moore-Colyer et al*.,* 2014; Stockdale and Moore-Colyer 2010). Blumerich et al. (2012) supported this by reporting that feeding steamed hay to sEA-affected horses reduced clinical score and total airway neutrophilia (Blumerich et al., 2012), both current diagnostic techniques of sEA in horses. Furthermore, in mild-moderate equine asthma, feeding steamed hay reduced the chances of disease and fungal particles found in tracheal washes compared with soaking hay or haylage (Deauville *et al.,* 2018). However, the exact methods behind this remain unclear. One potential mechanism is the denaturation of proteins via thermal methods, preventing IgE recognition. Research in this area is however contradictory, demonstrating that in some proteins the allergenicity is completely destroyed, whereas many remain completely unaltered or may in fact be increased. The impact thermal processing will have on the allergen completely relies on the protein structure and stability, as well as the structure of the feedstuff in which the allergen is present (Mills et al., 2009). There are many examples of reduction in allergenicity by heating, but heat-denatured proteins can present new allergenic-sites uncovered in the unfolding process, and therefore is not a sufficient process when used solely to produce hypoallergenic foodstuffs (Davis and Williams, 1998). These results demonstrate that steaming in the

HAYGAIN 600 hay steamer has no effect on IgE-protein binding, but it has recently been demonstrated that hay steaming reduces fungal particles in tracheal washes, whereas soaking hay did not (Dauvillier et al., 2018). *Ergo,* the mechanisms behind the apparent efficacy of hay steaming is likely to result from; (1) decreased respirable particles; or (2) protein coagulation "fixing" potential allergenic proteins to the hay. Either way, the methods behind which steaming reduces the clinical signs appears to be through reducing allergen:animal interaction, rather than any significant alteration in allergenic epitopes.

The results demonstrated some instances in which hay was not the predominant allergen source, this could be a result of sensitisation resulting from a previous season of hay, or from another environmental source, such as bedding or circulating pollen. This microarray platform proved to effectively identify allergens present in the horse's hay, thus demonstrating the technique's ability to identify allergens in environmental samples associated with the stabling environment. The same method could be applied for the assessment of bedding and swab samples of stables. Therefore, this technique has great potential in assisting the formulation of effective allergen-avoidance programs. However, it is limited to allergens included on the assay, and the allergens to which a specific horse is sensitised. The results highlighted that hay is a major allergen source in sEA-affected horses regardless of treatment. The best allergen-avoidance regimes should therefore exclude hay. However, this is clearly not a viable option for many owners. In which case, this microarray platform could be used to identify a

supply of hay with minimal allergens to which the horse is sensitised. Furthermore, the hay must undergo treatment to reduce respirable particulate matter. Several researchers have established that steaming is equally effective in reducing respirable dust particles to soaking, but preferable to the latter when considering bacteria content, reduction of respirable fungal fragments, and the unknown effect of allergen ingestion in eliciting symptoms in SEA-sensitized individuals (Blackman and Moore-Colyer, 1998; Blundell et al., 2012; Dauvillier et al., 2011; Tizard, 1977). Moreover, steaming has the added benefit of inactivating fungi and bacteria present, therefore preventing contamination of the stabling environment (e.g. bedding) leading to proliferation of potential allergens.

8.4 Limitations of the study and future prospective

As there is fairly limited research conducted into the microbial content of the ARP present in the stabling environment, it was a challenge to make a full and extensive decision of which proteins to select for inclusion in the protein microarray. Due to the nature of the method, it is therefore limited purely to the proteins included for assessment. In further studies 16S and ITS sequencing should be conducted to enable a true assessment of the bacterial and fungal content present in ARP within the stabling environment. Similarly, to keep as a wide an approach as possible in terms of protein inclusion, a number of protein extracts were used, meaning the sensitivity and specificity of the technique would not match that of one purely based on recombinant/pure proteins. To combat this, future studies should utilise sequencing data, and

extracts could be assessed and shortlisted to those of interest. From this, proteins could be fractioned and undergo a second shortlisting with those of interest being produced as recombinant proteins. Unlike other IgE-mediated conditions, such as IBH, sEA appears to have a large and varied sensitisation profile. In terms of on-site diagnostic developments this is quite limiting. Further work moving towards CRD as outlined above would most likely improve diagnostic accuracy, and therefore enable diagnostics using a more limited range of proteins.

The microarray profiling of sIgE had several limitations. A commonly considered pitfall in such IgE profiling work is IgG concentrations: this can result in IgG cross-binding to allergens, as well as creating competition for protein-binding and potential cross-reactivity, which may influence the quality of fluorescent signal where there are low levels of IgE present in the sample. This was navigated as much as reasonably possible by using the highly selective mouse anti-horse IgE mAb 3H10 clone. Due to the limited range of asthmatic research herds worldwide, few matched samples were sourced, moreover, the numbers present from each site were limited. This was unavoidable, and certainly the inclusion of environmentally controlled samples was important for the study but was still a limitation. If assessed on their own, the limited matched samples could result in type II errors, meaning complexities in classification, however this was not the case. Further variables included disease diagnosis and previous/common allergen exposure, which has previously been discussed. These variables were managed wherever possible and taken into account (e.g. comparative analysis of mixed and

matched groups). Most sEA horses are managed in a way that avoids allergen interaction, and thus exacerbation of their disease. This reduced exposure to the causative allergen could have resulted in decreased sIgE, as we found here when attempting to compete the French samples with their corresponding hay.

Sensitization profiles, particularly those derived from CRD, may be utilised in the development of hyposensitization treatments, such as AIT. This represents the only causative treatment for allergies. Utilising the sEA sensitization profiles, transgenic barley produced allergens would enable sublingual immunotherapy treatment, as seen in insect bite hypersensitivity (Jonsdottir et al., 2018). The development of AIT is largely dependent on the full panel of allergens having been elucidated and produced as recombinant proteins.
Furthermore, once the inciting allergens have been identified, it is possible to provide a tailored allergen avoidance regime. This might be highly beneficial in terms of preventative management, for example, if a horse is highly reactive to pollens, turnout can be avoided on days with particularly high pollen counts. Similarly, if a horse is particularly reactive to a certain strain of fungi, this can be actively assessed in forage and bedding prior to purchase, or when assessing air quality. This may, however, be limited, as may not be feasible for every owner on a strain-by-strain basis, but may be of value when considering an entire protein group (e.g. fungi). Nonetheless, this approach would support active allergen avoidance, which is the cornerstone of sEA treatment.

Hay/environmental analysis offers a hugely advantageous technique but is obviously limited to analysis of a specific horse's hypersensitivities and does not quantify the levels of a panel of allergen present in a fodder, merely if it is an allergen for a specific animal. As a diagnostic tool it is useful in the creation of allergen avoidance regimes. Once a further panel of common allergen have been established as sEA inciting, further work developing and employing capture antibodies against the specific causal allergen is needed, thus enabling wide scale and comprehensive analysis of a single environmental sample.

8.5 Research impact

Main findings of the research:

- Comprehensive microarray IgE profiling and mathematical modelling enabled the accurate classification of horses affected with severe equine asthma (sEA).
- The microarray revealed a plethora of novel pollen, bacteria, mould and arthropod proteins implicated in the aetiology of sEA.
- This work further implicates the role of IgE in sEA.
- Latex proteins, an allergen group previously untested in the horse, were the most influential variables.
- The microarray platform can be utilised as a competition method for the identification of allergen sources.

- Sensitization profiles can be utilised in the development of AIT, the only causative treatment for allergies.

Novelty of the work:

- The high discriminatory power of this approach enabled the accurate diagnosis of sEA using serological IgE as a biomarker for the first time, potentially preventing the necessity for sedation and more invasive diagnostic methods.
- The largest scale screening of sEA-associated environmental proteins to date.
- Revealed of a wide range of novel sEA-associated allergen.
- Identified exposure to latex should be considered a potential risk to the respiratory health of the horse.
- Produced a new assay to enable the evaluation of IgE-protein binding.
- Enables the creation of accurate allergen avoidance through a novel wide scale allergen source assessment.

8.6 References

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9 Conclusion

The developmental work clearly established a reliable protein microarray for large scale IgE profiling of equine environmental proteins, confirming identified sEA-associated allergens and elucidating a range of previously unidentified allergens. The technique is sufficiently sensitive and specific to differentiate between sensitised allergens in sEA and control horses. Furthermore, the developed serological assay enables accurate identification of an individual horse's sensitisation profile. This information provides a reliable, fast and repeatable method for screening a wide variety of potential allergens found in the stabling environment in a miniaturised and affordable format, while offering a platform to support management and treatment of this debilitating respiratory disorder in horses. More advanced assessment utilising the microarray platform demonstrated here can be used to identify allergens associated with sEA and may assist in the diagnosis of the disease. Serological investigation of 138 horses living in varying environments showed that sEA is associated with a large sensitisation profile, predominantly latex, fungi, mite and pollen proteins. These results have shown for the first time that exposure to latex may be considered a risk to the respiratory health of the horse. Sensitivity and specificity values confirm the high discriminatory power of the technique in combination with mathematical modelling. Moreover, the microarray platform described here can be used to assess the presence of sEA-associated allergen in hay and assist in the creation of allergen-avoidance programs. This technique could have future application in environmental testing, such as bedding and environmental swabs. These results would suggest that current hay treatment methods have no effect on IgE-protein binding and that

preventing allergen inhalation is the best solution for sEA affected horses. However, where this is not possible, steaming hay before consumption is advisable due to decreased allergen exposure, and inactivation of mould and bacteria. The novel competitive microarray platform demonstrated here will enhance the health, welfare and performance of sEA-affected horses by enabling accurate environmental allergen testing and the implementation of effective avoidance strategies. This has been achieved on a number of levels through (a) the development of a novel serological diagnostic test, (b) improved understanding of disease pathogenesis, and (c) identification of novel allergenic candidates.

Appendix A: paper I supplementary data

Genus species	Allergome Name	Groups
Actinidia deliciosa	Act d 1	fruit
Alnus glutinosa	Aln g	pollen
Alnus glutinosa	Aln g	pollen
Ambrosia artemisiifolia	Amb a 1	pollen
Anisakis simplex	Ani s 1	nematode
Anisakis simplex	Ani s 3	nematode
Apis mellifera	Api m 1	artropode
Apis mellifera	Api m 4	artropode
Arachis hypogaea	Ara h 1	nut
Arachis hypogaea	Ara h 2	nut
Arachis hypogaea	Ara h 6	nut
Artemisia vulgaris	Art v 4	pollen
Aspergillus fumigatus	rAsp f 1	fungus
Aspergillus fumigatus	rAsp f 3	fungus
Aspergillus fumigatus	rAsp f 4	fungus
Aspergillus fumigatus	rAsp f 6	fungus
Aspergillus fumigatus	rAsp f 8	fungus
Bertholletia excelsa	Ber e 1	nut
Betula verrucosa	Bet v 1.0101	pollen
Blattella germanica	Bla g 1	artropode
Blattella germanica	Bla g 2	artropode
Blattella germanica	Bla g 4	artropode

Blattella germanica	Bla g 5	artropode
Bos domesticus	Bos d 4	milk
Bos domesticus	Bos d 5	milk
Bos domesticus	Bos d 6	milk
Bos domesticus	Bos d 8	milk
Canis familiaris	Can f 1	animal
Canis familiaris	Can f 3	animal
Carpinus betulus	Car b	seed
Corylus avellana	Cor a 1.0103	pollen
Corylus avellana	Cor a 9	nut
Cupressus arizonica	Cup a 1	pollen
Cynodon dactylon	Cyn d 12	pollen
Dermatophagoides farinae	Der f 1	artropode
Dermatophagoides farinae	Der f 2	artropode
Dermatophagoides pteronyssinus	Der p 1	artropode
Dermatophagoides pteronyssinus	Der p 10	artropode
Dermatophagoides pteronyssinus	Der p 2	artropode
Dermatophagoides pteronyssinus	Der p 7	artropode
Equus caballus	Equ c 3	animal
Euroglyphus maynei	Eur m 2	artropode
Felis domesticus	Fel d 2	animal

Fraxinus excelsior	Fra e	pollen
Gallus domesticus	Gal d 1	egg
Gallus domesticus	Gal d 2	egg
Gallus domesticus	Gal d 3	egg
Gallus domesticus	Gal d 4	egg
Glycyphagus domesticus	Gly d 2	artropode
Helix aspersa	Hel as 7	mollusco
Hevea brasiliensis	Hev b 1	latex
Hevea brasiliensis	Hev b 10	latex
Hevea brasiliensis	Hev b 11	latex
Hevea brasiliensis	Hev b 6.02	latex
Hevea brasiliensis	Hev b 7.02	latex
Hevea brasiliensis	Hev b 8	latex
Hevea brasiliensis	Hev b 9	latex
Lolium perenne	Lol p 1	pollen
Mercurialis annua	Mer a 1	fish
Mus musculus	Mus m 1	animal
Olea europaea	Ole e 1	pollen
Olea europaea	Ole e 2	pollen
Parietaria judaica	Par j 1	pollen
Parietaria judaica	Par j 2	pollen
Parietaria judaica	Par j 3	pollen
Penaeus indicus	Pen i 1	crustacea
Periplaneta americana	Per a 7	artropode
Phleum pratense	Phl p 1.0102	pollen

Phoenix dactylifera	Pho d 2	pollen
Platanus acerifolia	Pla a 1	pollen
Platanus acerifolia	Pla a 2	pollen
Prunus persica	Pru p 3	fruit
Rattus norvegicus	Rat n 1	animal
Solanum tuberosum	Sola t 1	tuber
Triticum aestivum	Tri a Gliadin	seed
Ananas comosus	Ana c 2	fruit
Carica papaya	Car p 1	fruit
Populus alba	Pop a	pollen
Eucalyptus globulus	Euc g [Pollen]	pollen
Alternaria alternata	Alt a 1	fungus
Actinidia deliciosa	Act d 2	fruit
Artemisia vulgaris	Art v 1	pollen
Helianthus annuus	Hel a 2S Albumin	seed
Platanus acerifolia	Pla a 8	pollen
Armoracia rusticana	Arm r HRP	tuber
Malassezia pachydermatis	Mala p	fungus
Linum usitatissimum	Lin us	pollen
Triticum polonicum	Tri tp	seed
Triticum turgidum ssp. durum	Tri td	seed
Lupinus albus	Lup a [Seed]	seed
Culex pipiens	Cul p	artropode
Penaeus monodon	Pen m 1	crustacea
Bos domesticus	Bos d LF	milk

Drechslera spicifera	Dre s	fungus
Glycine max	Gly m TI	seed
Aedes communis	Aed c	artropode
Aspergillus versicolor	Asp v	fungus
Quercus ilex	Que i	pollen
Glycine max	Gly m Agglutinin	seed
Malus domestica	Mal d 1.0108	fruit
Leucanthemum vulgare	Leu vu	pollen
Rhizopus nigricans	Rhi n	fungus
Acarus siro	Aca s	artropode
Agrostis gigantea	Agr g [Pollen]	pollen
Alnus glutinosa	Aln g	pollen
Alternaria alternata	Alt a	fungus
Ambrosia artemisiifolia	Amb a	pollen
Ambrosia psilostachya	Amb p	pollen
Anisakis simplex	Ani s	nematode
Anthoxanthum odoratum	Ant o [Pollen]	pollen
Apium graveolens	Api g [Root]	vegetable
Apis mellifera	Api m [Venom]	artropode
Artemisia vulgaris	Art v	pollen
Aspergillus fumigatus	Asp f	fungus
Aspegillus flavus	Asp fl	fungus
Aspergilus niger	Asp n	fungus
Aspergillus nidulans	Aspe ni	fungus
Avena sativa	Ave s [Pollen]	pollen

Betula verrucosa	Bet v [Pollen]	pollen
Blattella germanica	Bla g	artropode
Blatta orientalis	Bla o	artropode
Blomia tropicalis	Blo t	artropode
Bos domesticus	Bos d [Milk]	milk
Brassica	Bra n	Brassica
Bromus inermis	Bro I [Pollen]	pollen
Canis familiaris	Can f [Epithelium]	animal
Candida albicans	Cand a	fungus
Castanea sativa	Cas s [Pollen]	nut
Chenopodium album	Che a	pollen
Cladosporium herbarum	Cla h	fungus
Corylus avellana	Cor a [Pollen]	pollen
Ctenocephalides felis	Cte f	artropode
Culicoides nubeculosus	Cul n	culicidae
Cupressus arizonica	Cup a [Pollen]	pollen
Cynodon dactylon	Cyn d [Pollen]	pollen
Dactylis glomerata	Dac g [Pollen]	pollen
Daucus carota	Dau c	tuber
Dermatophagoides farinae	Der f	artropode
Dermatophagoides pteronyssinus	Der p	artropode
Epicoccum nigrum	Epi p	fungus
Equus caballus	Equ c [Epithelium]	animal
Euroglyphus maynei	Eur m	artropode

Fagopyrum esculentum	Fag e	seed
Felis domesticus	Fel d [Epithelia]	animal
Festuca pratensis	Fes p [Pollen]	pollen
Fusarium solani	Fus s	fungus
Gallus domesticus	Gal d [Egg White]	egg
Glycyphagus domesticus	Gly d	artropode
Glycine max	Gly m	seed
Glycine max	Gly m 4	seed
Helianthus annuus	Hel a [Pollen]	seed
Hevea brasiliensis	Hev b	latex
Holcus lanatus	Hol l [Pollen]	pollen
Hordeum vulgare	Hor v [Pollen]	pollen
Juniperus virginiana	Jun v	pollen
Lepidoglyphus destructor	Lep d	artropode
Lolium perenne	Lol p [Pollen]	pollen
Malus domestica	Mal d [Fruit]	fruit
Musa x paradisiaca	Mus xp	fruit
Olea europaea	Ole e [Pollen]	pollen
Parietaria judaica	Par j	pollen
Parietaria officinalis	Par o [Pollen]	pollen
Penicillium chrysogenum	Pen ch	fungus
Periplaneta americana	Per a	artropode
Phleum pratense	Phl p	pollen
Platanus acerifolia	Pla a [pollen]	pollen
Plantago lanceolata	Pla l	pollen

Poa pratensis	Poa p	pollen
Saccharomyces cerevisiae	Sac c	fungus
Salsola kali	Sal k	pollen
Secale cereale	Sec c [Pollen]	pollen
Sorghum halepense	Sor h	pollen
Triticum aestivum	Tri a [Seed]	seed
Tyrophagus putrescentiae	Tyr p	artropode
Vespa crabro	Vesp c	artropode
Zea mays	Zea m [Pollen]	pollen
Bos domesticus	Bos d [Epithelium]	milk
Castanea sativa	Cas s [Seed]	nut
Corylus avellana	Cor a [Seed]	nut
Equus caballus	Equ c [Milk]	animal
Gallus domesticus	Gal d [Egg Yolk]	egg
Helianthus annuus	Hel a [Seed]	seed
Hordeum vulgare	Hor v [Seed]	seed
Mus musculus	Mus m [Epithelium]	animal
Oryza sativa	Ory s [Seed]	seed
Ovis aries	Ovi a [Epithelium]	animal
Rattus norvegicus	Rat n [Epithelium]	animal
Ricinus communis	Ric c [Pollen]	pollen
Triticum aestivum	Tri a [Pollen]	pollen
Zea mays	Zea m [Seed]	seed
Acer saccharinum	Ace s	pollen
Aureobasidium pullulans	Aur p	fungus

Bipolaris sorokiniana	Bip so	fungus
Chaetomium globosum	Cha g	fungus
Corylus americana	Cor am	nut
Culicoides obsoletus	Cul s	culicidae
Eupatorium capillifolium	Eup c	pollen
Eurotium amstelodami	Eur s	fungus
Fagus grandifolia	Fag g	pollen
Geotrichum candidum	Geo c	fungus
medicago sativa	Med s [Pollen]	pollen
Penicillium digitatum	Pen d	fungus
Penicillium expansum	Pen e	fungus
Quercus robur	Que r [Pollen]	pollen
Rumex acetosella	Rum a	pollen
Rumex crispus	Rum cr	pollen
Salix lasiolepis	Sal la	pollen
tripholium pratense	Tri pr	vegetable
Ulmus americana	Ulm a	pollen
Urtica dioica	Urt d [Pollen]	pollen
Cladosporium herbarum	Cla h	fungus
Bos domesticus	Bos d 9	milk
Bos domesticus	Bos d 11	milk
Bos domesticus	Bos d 12	milk
Bos domesticus	Bos d 5.0102	milk
Actinidia deliciosa	Act d 5	fruit
Anisakis pegreffii	Ani pe	nematode

Aspergillus restrictus	Asp r 1	fungus
Alternaria alternata	Alt a 6.0101	fungus
Betula verrucosa	Bet v 2.0101	pollen
Hevea brasiliensis	Hev b 3.0101	latex
Hevea brasiliensis	Hev b 5.0101	latex
Salsola kali	Sal k 1	pollen
Phleum pratense	Phl p 12.0101	pollen
Phleum pratense	Phl p 2.0101	pollen
Phleum pratense	Phl p 6.0101	pollen
Phleum pratense	Phl p 7.0101	pollen
Drechslera halodes	Dre h	fungus
Thermoactinomyces vulgaris	The v	Bacteria
Saccharopolyspora rectivirgula	Sac r	Bacteria
Actinidia chinensis	Act c 5	fruit
Actinidia chinensis	Act c 10	fruit
Actinidia deliciosa	Act d 10	fruit
Dermatophagoides pteronyssinus	Der p 23	artropode
Actinidia deliciosa	Act d 11	fruit
Taraxacum officinale	Tar o [Pollen]	pollen
Black fly	Sim vi	artropode
Amaranthus cruentus	Ama cr	pollen
Merluccius merluccius	Mer mr 1	fish
Merluccius hubbsi	Mer hu 1	fish
Merluccius capensis	Mer ca 1	fish

Solanum lycopersicum	Sola l	seed
Corylus avellana	Cor a PG	nut
Actinidia chinensis	Act c 11	fruit
Ovis aries	Ovi a [wool]	animal
Prunus persica	Pru p 7	fruit
Prunus persica	Pru p Hevb5-like	fruit
Mucor circinelloides	Muc ci	fungus
Culicoides obsoletus	Cul ob	culicidae
Equus caballus	Equ c Myoglobin	animal
Bos domesticus	Bos d CA	milk
Actinidia chinensis	Act c Chitinase_IV	fruit
Ceratonia siliqua	Cer si [Seed]	seed
Prunus persica	Pru p 3	fruit
Zea mays	Zea m [Seed]	seed
Avena sativa	Ave s [Seed]	seed
Secale cereale	Sec c [Seed]	seed
Triticum aestivum	Tri a [Seed]	seed
Arachis hypogaea	Ara h 1-NT	nut
Arachis hypogaea	Ara h 1-CT	nut
Glycine max	Soybean AL3- native 1mg/ml	seed
Glycine max	Soybean AL3	seed
Glycine max	Soya 11S	seed
Lepidoptera	Moth Heterocera	artropode
Controls	IgE	controls

Controls	IgE	controls
Controls	Cy5	controls
Controls	Cy3	controls
Controls	PBS	controls
Helix aspersa	Hel as 7	mollusco
Periplaneta americana	Per a 7	artropode
Zea mays	Zea m [Seed]	seed
Setosphaeria rostrata	Set r	fungus
Kineosporia rhizophila	Kin r	Bacteria
Acinetobacter gerneri	Aci g	Bacteria
Aeromicrobium ponti	Aer p	Bacteria
Rhodococcus kroppenstedtii	Rhod k	Bacteria
Streptomyces albus	Str a	fungus
Microbacterium marinilacus	Mic m	Bacteria
Erwinia rhapontici	Erw r	Bacteria
Culicoides nubeculosus	Cul n 1	culicidae
Culicoides nubeculosus	Cul o 3	culicidae
Culicoides nubeculosus	Cul n 2	culicidae
Culicoides nubeculosus	Cul n 3	culicidae
Culicoides nubeculosus	Cul n 4	culicidae
Culicoides nubeculosus	Cul n 8	culicidae
Culicoides nubeculosus	Cul n 5	culicidae
Culicoides nubeculosus	Cul o 7	culicidae
Culicoides obsoletus	Cul o 2	culicidae
Culicoides obsoletus	Cul n 4	culicidae

Culicoides obsoletus	Cul o 3	culicidae
Culicoides obsoletus	Cul o 3	culicidae
Culicoides obsoletus	Cul o 2	culicidae
Culicoides obsoletus	Cul n 1	culicidae
Culicoides obsoletus	Cul n 2	culicidae
Culicoides obsoletus	Cul o 2	culicidae
Culicoides obsoletus	Cul n 3	culicidae
Culicoides obsoletus	Cul n 4	culicidae
Culicoides obsoletus	Cul o 7	culicidae
Culicoides obsoletus	Cul nu 2	culicidae
Culicoides nubeculosus	Cul nu 10.01	culicidae
Culicoides nubeculosus	Cul nu 10.02	culicidae
Culicoides nubeculosus	Cul nu 4	culicidae
Culicoides obsoletus	Cul ob 8	culicidae
Culicoides nubeculosus	Cul ob 8	culicidae
Culicoides nubeculosus	Cul ob 8	culicidae
Culicoides obsoletus	Cul nu 4	culicidae
Culicoides obsoletus	Cul nu 4	culicidae
Culicoides obsoletus	Cul nu 2	culicidae
Culicoides obsoletus	Cul n 10.03	culicidae
Culicoides obsoletus	Cul nu 2	culicidae
Culicoides nubeculosus	Cul o 7	culicidae
Culicoides nubeculosus	cul o 2	culicidae
Culicoides nubeculosus	cul o 2	culicidae
Culicoides nubeculosus	Cul o 1	culicidae

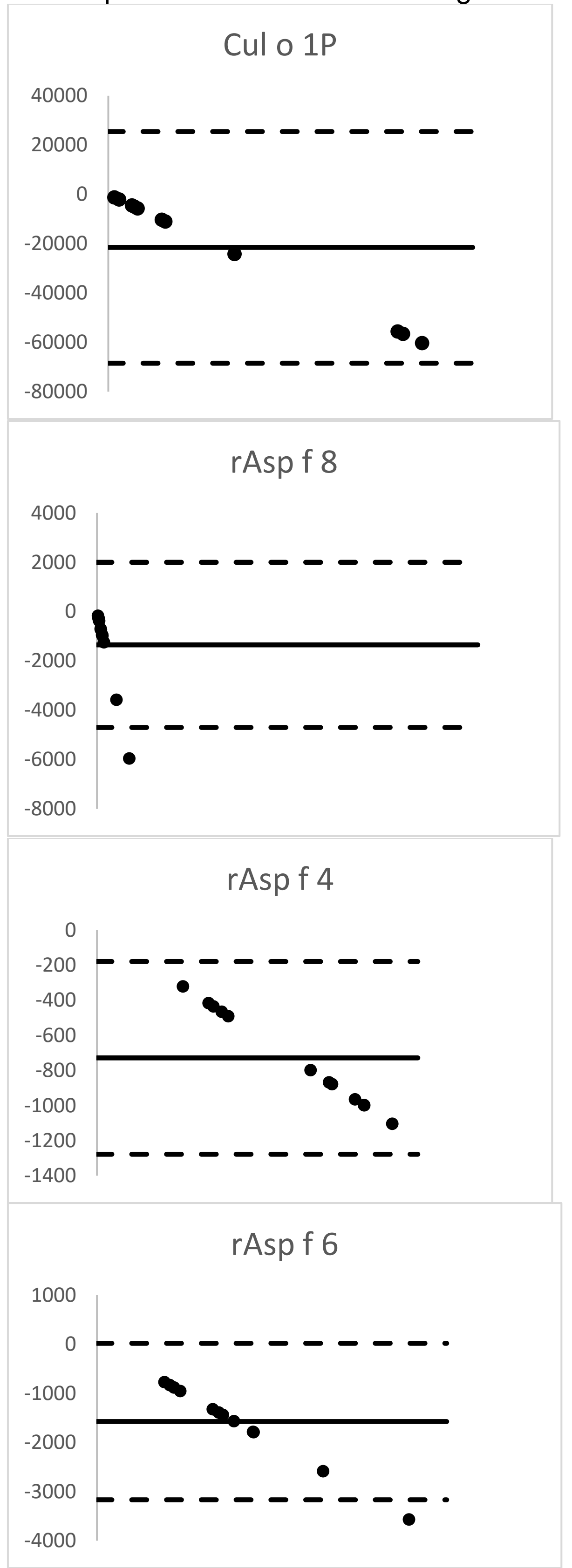
Culicoides	Cul o 3	culicidae
Culicoides	Cul o 4	culicidae
Culicoides	cul o 5	culicidae
Culicoides	Cul o 6	culicidae
Culicoides	Cul o 7	culicidae
Culicoides	Cul o 8	culicidae
Culicoides nubeculosus	Cul o1P	culicidae
Alternaria alternata	Alt a 1	fungus
Culicoides obsoletus	Cul o 5	culicidae
Culicoides obsoletus	Cul o 6	culicidae
Culicoides obsoletus	C015	culicidae
Culicoides obsoletus	C0120	culicidae
Culicoides obsoletus	C0145	culicidae
Culicoides obsoletus	C0147	culicidae
Culicoides obsoletus	C0180	culicidae
Culicoides nubeculosus	Cul nu 1	culicidae
Culicoides nubeculosus	Cul nu 1	culicidae
Culicoides nubeculosus	Cul nu 2	culicidae
Culicoides nubeculosus	Cul nu 2	culicidae
Culicoides nubeculosus	Cul nu 3	culicidae
Culicoides nubeculosus	Cul ob 3	culicidae
Culicoides nubeculosus	Cul nu 4	culicidae
Culicoides nubeculosus	Cul nu 6-like	culicidae
Culicoides nubeculosus	Cul nu 6-like	culicidae
Culicoides obsoletus	Cul ob 6.01	culicidae

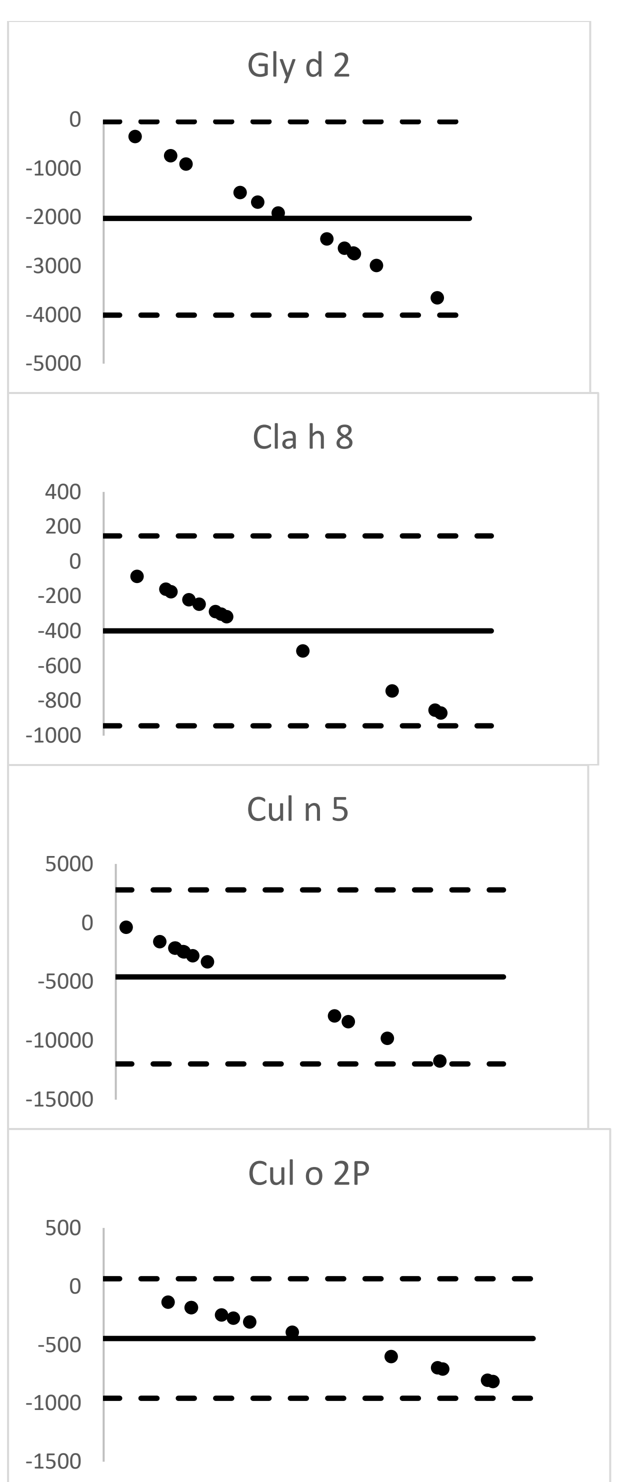
Culicoides obsoletus	Cul ob 6.01	culicidae
Culicoides obsoletus	Cul ob 6.02	culicidae
Culicoides obsoletus	Cul ob 6-like	culicidae
Culicoides obsoletus	Cul ob 6-like	culicidae
Culicoides obsoletus	Cul ob 8	culicidae
Culicoides nubeculosus	Cul nu 9	culicidae
Culicoides nubeculosus	Cul nu 9	culicidae
Culicoides nubeculosus	Cul nu 10.01	culicidae
Culicoides nubeculosus	Cul nu 10.01	culicidae
Culicoides nubeculosus	Cul nu 10.02	culicidae
Culicoides nubeculosus	Cul nu 10.03	culicidae
Culicoides nubeculosus	Cul nu 10.03	culicidae
Culicoides nubeculosus	Cul n 11	culicidae
Culicoides nubeculosus	Cul nu 12	culicidae
Culicidae	Culicidae Cul A	culicidae
Culicidae	Culicidae Cul B	culicidae
Culicidae	Culicidae Cul C	culicidae
Culicidae	Culicidae Cul D	culicidae
Culicidae	Culicidae Cul E	culicidae
Culicidae	Culicidae Cul F	culicidae
Culicoides nubeculosus	Cul n	culicidae
Alternaria alternata	Alt a 1	culicidae
Hay dust	Hay d	Hay related
Culicoides obsoletus	Co 13	culicidae
Culicoides obsoletus	Co 60	culicidae

Culicoides obsoletus	Co167	culicidae
Culicoides obsoletus	Cul o 2	culicidae
Culicoides obsoletus	Cul o 3	culicidae
Culicoides obsoletus	Cul o 3	culicidae
Culicoides obsoletus	Cul o 5	culicidae
Culicoides obsoletus	Cul o 6 F10	culicidae
Culicoides obsoletus	Cul o 6 F10	culicidae
Culicoides obsoletus	Cul o 8 C11	culicidae
Bos domesticus	Bos d LF	milk
Bos domesticus	Bos d 5	milk
Bos domesticus	Bos d 4	milk
Culicoides obsoletus	Cul o 2	culicidae
Culicoides obsoletus	Cul o 3	culicidae
Culicoides obsoletus	cul o 5	culicidae
Culicoides obsoletus	Cul o 6F10	culicidae
Culicoides obsoletus	Cul o 8C11	culicidae
Manioc	Man e	tuber
Manioc	Man e	tuber
Dermatophagoides farinae	Der f	artropode

Appendix B: paper II supplementary data

Bland Altman plots to compare ELISA and microarray IgE with specific proteins. The acceptable level of bias is dictated by the solid line. The dashed lines represent the 95% limits of agreement as the mean difference (2 SD).




Gly d 2
0
-1000
-2000
-3000
-4000
-5000
Cla h 8
400
200
0
-200
-400
-600
-800
-1000
Cul n 5
5000
0
-5000
-10000
-15000
Cul o 2P
500
0
-500
-1000
-1500

Summary of horses used in the study.

	Switzerland	**France**	**USA**	**Canada**
Number of horses	16 sEA affected 24 IBH affected 23 IBH/sEA affected 40 control	5 sEA affected 6 control	6 sEA affected 6 control	6 sEA affected 6 control
Horse Owner Assessed Respiratory Signs Index (HOARSI) (Ramseyer et al., 2007)	†	na	na	na
Histamine/Metacholin Bronchoprovocation : (concentration of histamine when inhalation was stopped)	†	na	na	na
Partial pressure of arterial oxygen	†	na	na	na
Provocation concentration	†	na	na	na
Pulmonary function test	†	†	†	†
Insect bite hypersensitivity score (Etude, 2014)	†	na	na	na
Physical examination : including respiratory and cardiac auscultation, rectal temperature, capillary refill time and colour of mucous membranes, palpation of submandibular lymph nodes, lung auscultation at rest and during rebreathing	†	†	†	†
Mucus scoring system	na	†	†	†
Bronchoalveolar lavage fluid (including cytology)	na	†	†	†
Owner reported coughing, nasal discharge at time of examination	†	†	†	†
Reversible airway obstruction (after medical/environmental change)	na	†	na	na

† : measured in this group
na : not analysed

Variable influences on the projection scores significant for class prediction from the environmentally mixed group of horses (n=138) after VIP selection.

Species	Allergome name	Extract = E; Molecule = M	VIP score
Hevea brasiliensis	Hev b 11	M	2.397886012
Hevea brasiliensis	Hev b 6.02	M	2.242175574
Hevea brasiliensis	Hev b 5.0101	M	2.061708356
Aspergillus fumigatus	rAsp f 8	M	2.034462948
Helix aspersa	Hel as 7	M	1.693216892
Culicoides obsoletus	Cul o 145	M	1.553348148
Hevea brasiliensis	Hev b 3.0101	M	1.447433432
Betula verrucosa	Bet v 2.0101	M	1.439197666
Culicoides obsoletus	Cul nu 2	M	1.401734766
Culicoides nubeculosus	Cul o1P	M	1.370674276
Blattella germanica	Bla g 2	M	1.294109254
Culicoides obsoletus	Cul o 2	M	1.289809746
Mercurialis annua	Mer a 1	M	1.257180767
Bos domesticus	Bos d LF	M	1.234823184
Culicoides obsoletus	Cul ob 8	M	1.132147319
Prunus persica	Pru p 3	M	1.131899686
Felis domesticus	Fel d [Epithelia]	E	1.131671083
Mucor circinelloides	Muc ci	E	1.113604072
Culicoides obsoletus	C0120	M	1.107017518
Quercus robur	Que r [Pollen]	E	1.103588161
Culicoides obsoletus	Cul ob 8	M	1.10169779
Epicoccum nigrum	Epi p	E	1.097080342
Gallus domesticus	Gal d [Egg White]	E	1.092020409
Thermoactinomyces vulgaris	The v	E	1.078503603
Culicoides obsoletus	Cul n 10.03	M	1.070733631
Hevea brasiliensis	Hev b	E	1.053235133
Ulmus americana	Ulm a	E	1.046124579
Eupatorium capillifolium	Eup c	E	1.041304179
Actinidia deliciosa	Act d 2	M	1.039211209
Glycine max	Soybean AL3-recombinant	M	1.036586734
Parietaria judaica	Par j 1	M	1.025947792
Blomia tropicalis	Blo t	E	1.024506004
Rattus norvegicus	Rat n [Epithelium]	E	1.022811408
Corylus americana	Cor am	E	1.00432009
Culicoides nubeculosus	Cul ob 8	M	0.999496997
Lupinus albus	Lup a [Seed]	E	0.990564278
Culicoides obsoletus	Co167	M	0.983835139

Culicoides obsoletus	Cul o 8C11	M	0.98257687
Eurotium amstelodami	Eur s	E	0.981731287
Penaeus monodon	Pen m 1	M	0.977846295
Zea mays	Zea m [Seed]	E	0.975934894
Kineosporia rhizophila	Kin r	E	0.96268091
Olea europaea	Ole e 1	M	0.957886893
Aspegillus flavus	Asp fl	E	0.954692807
Olea europaea	Ole e [Pollen]	E	0.946553558
Dermatophagoides farinae	Der f 1	M	0.943956016
Geotrichum candidum	Geo c	E	0.934810722
Arachis hypogaea	Ara h 6	M	0.931278284
Helix aspersa	Hel as 7	M	0.929469269
Gallus domesticus	Gal d 4	M	0.92158607
Parietaria judaica	Par j	E	0.919641275
Culicoides	cul o 5	M	0.915549349
Helianthus annuus	Hel a 2S Albumin	M	0.915519266
Canis familiaris	Can f [Epithelium]	E	0.914611185
Solanum lycopersicum	Sola l LTP_7kD	M	0.909716836
Helianthus annuus	Hel a [Pollen]	E	0.898832645
Periplaneta americana	Per a 7	M	0.892563113
Corylus avellana	Cor a [Seed]	E	0.877063468
Culicoides obsoletus	Cul o 3	M	0.87297805
Dermatophagoides pteronyssinus	Der p 2	M	0.866653724
Olea europaea	Ole e 2	M	0.859525551
Fagopyrum esculentum	Fag e	E	0.859249296
Culicoides obsoletus	Cul o 6 F10	M	0.856052813
Eucalyptus globulus	Euc g [Pollen]	E	0.854059354
Quercus ilex	Que i	E	0.842572502
Dermatophagoides pteronyssinus	Der p	E	0.836111501
Actinidia chinensis	Act c 10	M	0.829294987
Artemisia vulgaris	Art v 1	M	0.824564544
Black fly	Sim vi	E	0.823110421
Ricinus communis	Ric c [Pollen]	E	0.815160266
Drechslera halodes	Dre h	E	0.807378063
Culicoides nubeculosus	Cul o 1	M	0.799266712
Hevea brasiliensis	Hev b 9	M	0.796595977
Aspergillus versicolor	Asp v	E	0.796427599
Avena sativa	Ave s [Seed]	E	0.790808149
Actinidia chinensis	Act c 11	M	0.787068315
Manioc	Man e	E	0.785608616
Glycine max	Gly m Agglutinin	M	0.78479311
Glycine max	Gly m TI	M	0.781228253

Periplaneta americana	Per a 7 (0.5mg)	M	0.779612014
Mus musculus	Mus m 1	M	0.777665393
Culicoides obsoletus	Cul o 3	M	0.776157103
hay dust	Hay d	E	0.774529898
Ceratonia siliqua	Cer si [Seed]	E	0.767985414
Culicoides nubeculosus	Cul nu 12	M	0.765522432
Blatta orientalis	Bla o	E	0.760381536
Castanea sativa	Cas s [Pollen]	E	0.748034147
Armoracia rusticana	Arm r HRP	E	0.740446527
Penicillium expansum	Pen e	E	0.734560812
Ctenocephalides felis	Cte f	E	0.716428089
Phleum pratense	Phl p 1.0102	M	0.712552232
Hevea brasiliensis	Hev b 8	M	0.710739268
Merluccius hubbsi	Mer hu 1	M	0.71046373
Dermatophagoides pteronyssinus	Der p 10	M	0.7096095
Hevea brasiliensis	Hev b 1	M	0.707560234
Taraxacum officinale	Tar o [Pollen]	E	0.700310884
Acarus siro	Aca s	E	0.698592024
Culicoides	Cul o 6	M	0.686143673
Bos domesticus	Bos d 5.0102	M	0.672595402
Platanus acerifolia	Pla a 8	M	0.669199521
Musa x paradisiaca	Mus xp	E	0.660557
Equus caballus	Equ c [Milk]	E	0.641591098
Cladosporium herbarum	Cla h	E	0.627312615
Triticum aestivum	Tri a [Pollen]	E	0.625247924
Canis familiaris	Can f 1	M	0.61595743
Saccharomyces cerevisiae	Sac c	E	0.600429374
Culicoides obsoletus	Cul nu 2	M	0.598849656
Carpinus betulus	Car b	E	0.571083305
Arachis hypogaea	Ara h 1	M	0.559353341
Alternaria alternata	Alt a 1	M	0.549001691
Leucanthemum vulgare	Leu vu	E	0.505030455
Brassica	Bra n	E	0.496039753
Acer saccharinum	Ace s	E	0.343599366

Individual approval numbers for sampling of horses per cohort. All samples used were surpluss to previous research requirements, and was approved by the the Royal Agricultural University Ethical Review Group.

Sample group	Applicant	Consenting body	Approval number	Previously published
France	Dr E. Richard	Comité d'éthique en recherche clinique et épidémilogique vétérinaire d'Oniris	CERVO-2017-8-V	Orard et al., 2018
Switzerland	Prof V. Gerber	Animal Experimental Committees of the Cantons of Berne, Freiburg, Solothurn, and Geneva, Switzerland	BE10/13; 58/10; 118/16	Verdon et al., 2018
Canada	Prof J. Lavoie	University of Montreal (animal experiment authorization number)	Rech-1578	Ghosh et al., 2016
USA	Prof L. Couetil	Purdue University Animal Care and Use Committee	A3471-01	Miskovic et al., 2007

Appendix C: paper III supplementary data

Supplementary Data

T-test results with Benjamini-Hochberg corrected *P*-values showing all statistically significant allergen between the sera samples and dry hay ($P = .05$)

Yard 1

Protein	**P value**
Sycamore Genuine marker Pla a 2	0.00273
Hazelnut Europenan orylus avellan Cor a [Pollen]	0.00316
Chestnut Pollen Cas s [Pollen]	0.00334
Rye Grass pollen genuine marker Lol p 1	0.00397
Barley Pollen Hor v [Pollen]	0.00482
Timothy phleum pratenses Phl p	0.00538
Malassezia Pachydermatis Mala p	0.00595
Juneky blue Poa pratensis Poa p	0.00624
Orchard Dactylis Glomerum Dac g [Pollen]	0.00648
Hazel pollen Polygalacturonase Cor a PG	0.00769
Aureobasidium Pullularia Pullus Aur p	0.00798
Corn Seed (Flour) Zea m [Seed]	0.0102
Penicilium notatum Pen ch	0.0111
Cy5 1mg/ml	0.0119
Oak pollen Que i	0.0129
Palm Profilin Pho d 2	0.0135
Rhizopus nigrican/stolo Rhi n	0.0137
Alternaria alternaria tem Alt a	0.0147
American Cockroah Tropomyosin Per a 7	0.0154
Tyrophagus putrescentiae Tyr p	0.0155
Aspergilus niger Asp n	0.0171
Artichoke Cyn s	0.0176
Johson grass Sorghum halepense Sor h	0.0200
Fennel dog Eupatorium ca Eup c	0.0205
Chaetomium globosum	0.0205
Kineosporia rhizophila	0.0242
Aspergillus genuine marker Asp r 1	0.0252
Setosphaeria rostrata	0.0278
Hazel nut extract Cor a [Seed]	0.0280
Helminthosporium spondylocladium Dre h	0.0284
Celery stalk Api g [Stalk]	0.0287
Snail Hel as	0.0306
broad bean extract Vic f	0.0307
soy bean extract Gly m	0.0323

Red top grass Agrostis gigantus (Pollen) Agr g [Pollen]	0.0338
Barley Seed Hor v [Seed]	0.0339
Corn Pollen Zea m [Pollen]	0.0343
Sunflower Pollen Hel a [Pollen]	0.0363
Shrimp Pan b	0.0363
Arroyo Willow Sal la	0.0400
Fescue meadow, Festuca pratense Fes p [Pollen]	0.0401
Rye, Perennial Lolium Perenne Lol p [Pollen]	0.0404
Cypress genuine marker Cup a 1	0.0405
Lamb quarter Chenopodium al Che a	0.0416
Common Sole Sol so	0.0438
Par j 2 Par j 2	0.0447
Wheat Pollen Tri a [Pollen]	0.0467
Yard 2	
Protein	P value
Wheat Gliadin fraction Tri a Gliadin	0.00106
Alternaria alternaria tem Alt a	0.00246
Aspergillus nidulans	0.00287
German cockroach genuine marker Bla g 2	0.00320
Der f 1 Der f 1	0.00706
Wheat Pollen Tri a [Pollen]	0.00849
Der p 1 Der p 1	0.0132
soy bean lectin Gly m Agglutinin	0.0141
Parietaria LTP Par j 1	0.0154
Aspergilus niger Asp n	0.0162
Malassezia Pachydermatis Mala p	0.0166
Cow's milk Bos d [Milk]	0.0168
Blood Amaranth Ama cr	0.0182
Helminthosporium spondylocladium Dre h	0.0183
Candida Cand a	0.0190
Nettle	0.0219
Asparagus Aspa o	0.0230
Cladosporium herbarum Cla h	0.0231
Rhizopus nigrican/stolo Rhi n	0.0237
Blue Mussel Myt e	0.0246
Penicilium notatum Pen ch	0.0268
Velvetgrass Holcus lanatus Hol l [Pollen]	0.0276
Margerite (Ox-eye) Chrysanthemum leu Leu vu	0.0294
Durum Wheat Tri td	0.0299
Mite genuine allergen Der p 7	0.0304
Aspergillus versicolor	0.0328
Oak pollen Que i	0.0339
Aspergillus fumigatus Asp f	0.0340
Geotrichum candidum	0.0349
Pru p 7 Pru p 7	0.0397

Johson grass Sorghum halepense Sor h	0.0398
SFA8 Hel a 2S Albumin	0.0410
Sweet Vernal grass Anthoxanthum Ant o [Pollen]	0.0426
Hazelnut Europenan orylus avellan Cor a [Pollen]	0.0437
Papain Car p 1	0.0457
Rice Ory s [Seed]	0.0471
Chestnut Pollen Cas s [Pollen]	0.0474
Lepidoglyphus Destructor Lep d	0.0499
Yard 3	
Protein	P value
Timothy phleum pratenses Phl p	0.00256
Aspergillus nidulans	0.00311
soy bean extract Gly m	0.00505
Aeromicrobium ponti	0.00558
Olive pollen genuine marler Ole e 1	0.00563
Tyrophagus putrescentiae Tyr p	0.00648
Cladosporium herbarum Cla h	0.00721
Cow's milk Bos d [Milk]	0.00767
Aureobasidium Pullularia Pullus Aur p	0.0111
Ragweed genuine marker Amb a 1	0.0162
Corn Seed (Flour) Zea m [Seed]	0.0177
Storage mite Eur m 2	0.0181
Acinetobacter gerneri	0.0205
Rhizopus nigrican/stolo Rhi n	0.0276
German cockroach genuine marker Bla g 1	0.0290
Snail Hel as	0.0327
Timothy pollen Phl p 7.0101	0.0340
Aspergilus niger Asp n	0.0346
Cow's milk Bos d [Milk]	0.0353
Cypress genuine marker Cup a 1	0.0379
Erwinia rhapontici	0.0394
Thermoactinomyces vulgaris	0.0400
Cladosporium herbarum	0.0411
Streptomyces albus	0.0412
Annual Mercury Profilin Mer a 1	0.0471
Velvetgrass Holcus lanatus Hol l [Pollen]	0.0497

Appendix D: Z-test raw data

sEA group

Name	AL2	AL2	AL11	AL11	AL21	AL21	AL22
Plantain english,	131	294	136	352	321	284	447
Cat Epithelia Fe	61	105	39	46	128	14	-27
Beech American	340	691	204	225	272	168	310
Helminthosporiu	2743	3806	473	374	617	637	1301
Moth Heterocera	452	708	386	398	1416	1586	900
Orchard Dactylis	382	428	439	542	306	317	504
Dock Yellow Rum	598	713	540	586	674	722	13666
ELM American Ul	310	265	270	295	203	138	326
Malassezia Pachy	764	949	288	394	1164	925	818
Flea Ctenocephal	169	22	37	49	11	62	13
Penicilium notatu	3346	3750	312	355	1679	1538	4058
Dandelion Taraxa	210	183	159	162	249	274	168
Bean castor Ric	148	320	228	279	249	251	366
Nettle Urtica Dio	138	154	112	167	132	120	220
Cy5	145	116	199	207	167	177	172
Mouse Epithelia	587	958	899	997	639	719	782
Rat epithelia Ra	575	635	733	772	618	674	651
Alternaria alterna	562	547	110	121	221	269	435
Johson grass Sorg	1278	1262	624	724	549	666	652
Birch allergopha	47	0	8	24	37	11	945
Cat allergopharm	34	7	22	67	23	26	-31
Horse Epithelia	385	316	366	320	385	435	221
Sheep's epithelia	174	81	149	168	121	159	168
Fennel dog Eupat	469	360	174	205	265	241	1284
Cypress, Arizona	418	369	108	86	523	550	277
Nettle	30	-7	30	30	2	7	-21
SFA8 Hel a 2S A	93	63	21	18	65	85	9
Euroglyphus May	1220	1157	430	458	3436	3804	633
Timothy phleum	436	488	427	483	390	402	521
Ragweed, Weste	109	141	121	132	123	124	559
Tyrophagus putre	7025	7620	991	1083	2180	2551	2133
Dermatophagoid	43	28	9	16	7	66	-9
Dermatophagoid	4	-43	-17	13	8	-16	54
IgE Neat	103	173	19	23	138	184	130
Fescue meadow,	567	543	462	516	335	300	491
Mugwort, Comm	265	218	170	222	287	311	162
Grass Smooth Bro	445	424	240	240	218	502	432
Der f 1 Der f 1	2851	2679	338	283	840	1051	2613
Der p 2 Der p 2	2336	2504	1118	1255	1014	1085	1848
Bermuda Cynodo	1067	944	712	918	744	779	9503
Rye, Perennial Lo	527	625	442	486	459	466	541
empty	-27	-6	28	9	3	3	-6
Poplar White Pop	407	211	292	243	357	302	279

Der f 2 Der f 2	1013	975	612	698	440	478	472
Der p 1 Der p 1	123	133	201	204	214	212	197
Red top grass Agr	703	649	489	426	386	403	697
Hazelnut America	1310	1524	371	457	615	553	334
Culicoides Culico	365	386	237	245	223	83	375
Maple soft Acer s	337	320	217	242	242	274	386
Eucalyptus Globu	143	141	128	144	166	172	176
Hazelnut America	1007	782	177	111	305	217	627
wool sheep ovis a	104	81	37	37	74	81	79
Cow Epitelia catt	313	309	268	305	349	300	350
Alder european A	715	869	340	401	333	294	512
Juneky blue Poa p	839	815	526	658	528	323	672
Cedar Red Junipe	380	463	182	212	304	569	155
Arroyo Willow S	437	379	373	402	465	474	479
Rhizopus nigrican	4786	4625	198	156	388	364	2169
Dog Epithelia C	488	523	451	411	420	456	421
Lamb quarter Ch	634	632	445	578	400	526	2004
Pellitory, wall Par	286	278	208	141	161	176	278
Cy5	228	169	245	208	263	164	155
empty	165	200	115	34	99	35	-9
Aspergilus niger	4038	4050	805	535	1533	1726	13161
Aureobasidium P	6106	5992	946	1233	2568	2736	2385
Poplar White Pop	358	359	233	227	339	350	312
Lepidoglyphus De	1705	1883	527	615	608	968	1126
Acarus Siro Acaru	54	245	56	56	46	61	239
Cladosporium he	371	555	71	41	82	137	587
Aspergillus fumig	2131	2306	565	422	1496	2114	2779
Blomia Tropicalis	3162	3273	711	818	1003	1114	1327
IgE half dilution	101	151	31	25	64	96	115
ragweed, Short A	493	301	297	289	315	411	628
Ber e 1 Ber e 1	129	57	21	27	191	30	33
Act c 1	88	94	128	478	104	105	132
Birch European B	111	340	339	332	229	261	196
Sycamore platanu	233	20	216	234	168	164	271
Rusian Thistle Sal	349	366	224	254	253	294	3893
Ash allergophar	1	-17	15	16	13	17	7
soy bean lectin	104	90	2168	2061	791	903	137
Soya 11S (Claire)	124	99	116	138	129	111	246
Velvetgrass Holcu	771	868	598	561	419	355	767
Oak English Quer	177	184	167	190	181	138	179
Cockroach, Germ	655	641	609	576	404	519	858
Rye Cultivated se	349	473	326	351	255	261	569
Phl p 1 Phl p 1.0	346	256	268	250	198	254	604
Phl p 12 Phl p 1	461	511	615	518	409	457	1039
Sweet Vernal gra	852	1097	720	718	650	419	844
Olive Olea europ	490	497	352	369	317	436	543
IgE one eight	38	125	45	110	-52	32	12

Cy5	428	456	432	413	309	375	381
Phl p 6 Phl p 6.0	3333	3489	1835	2139	1803	1948	5412
Phl p 2 Phl p 2.0	143	163	151	161	181	179	157
Sorrel red Rumex	29	28	4	49	37	-20	65
Margerite (Ox-ey	387	393	291	317	246	326	422
IgE one quarter	45	49	-8	5	-19	32	15
Mite genuine alle	1670	1311	656	765	1240	1035	2213
Lobster Norway	739	789	867	896	779	955	1868
Mosquito Aed c	316	258	303	383	345	370	427
Bet v 1a Bet v 1	786	714	795	780	704	710	1165
Bet v 2 Bet v 2.0	7127	7040	537	651	801	893	579
German cockroac	217	411	224	260	405	370	239
Storage mite Eu	387	282	179	183	32	81	233
Melon Cuc m [P	110	95	0	25	311	303	764
Monk fish (angler	82	110	177	193	1091	20	603
Par j 2 Par j 2	4570	4689	264	92	313	356	1203
rBer e 1 1mg/m	942	763	191	306	263	538	1865
Dog's Lipocalin	233	449	445	572	513	692	561
Apple Mal d [Fr	198	201	24	79	280	413	219
Orange Cit s [Fr	149	142	3	-33	195	383	163
Rabbit Dander O	616	703	847	1464	365	509	665
Human albumin	216	202	252	295	492	262	147
Human IgG 1mg	3009	3632	3228	3657	3204	2912	3218
Ananas (Pineappl	429	552	31	11	263	510	225
Artichoke Cyn s	371	338	152	135	275	701	440
Oyster Common	826	818	277	259	1606	1840	1477
Parietaria Par j	632	640	402	500	579	702	667
Cy3 1mg/ml	2213	1908	1858	2511	1529	1744	2221
Human IgA 1mg	3313	2926	4185	4636	3263	3334	3410
Anisakis (Oceans)	622	906	519	395	443	539	922
Chestnut Seed C	390	389	404	466	368	435	347
Sunflower Pollen	403	431	198	190	201	220	1056
Tunafish Thu a	70	3785	94	102	-11	21	127
DogIgG 10mg/m	6952	6019	7716	9144	5421	6607	8418
DogIgG 1mg/m	1641	1711	1624	1647	1159	1123	1476
celery extract A	111	126	-13	-21	90	588	59
Cockroach orient	1633	1431	524	940	972	1124	1899
Tiger Nut (Chufa)	733	737	316	895	424	486	855
Trout Onc m [M	-3	-20	-7	15	359	6	72
Dog IgM 1mg/m	432	342	363	482	370	341	601
Dog (?) IgE 10u	-36	-35	3	-4	33	-39	328
Chestnut Pollen	315	294	199	228	454	450	231
Hamster Epithelia	661	632	655	623	554	558	520
Anisakis genuine	478	479	393	375	493	458	424
Bermuda grass Pr	674	650	2296	1999	566	457	919
Peanut 7S Vicilin	341	334	201	370	407	165	517
Peanut 2S Album	300	333	107	112	137	234	74

Empty	20	15	-1	13	-37	-11	- 21
Hop pollen Hum	459	515	355	408	466	721	447
Anisakis Tropomy	391	355	420	618	457	339	994
Annual Mercury P	8036	9214	383	355	489	642	861
Ragweed genuine	452	446	324	342	282	360	921
Peanut 2S Album	10	60	41	24	31	44	39
Guinea pig Epithe	560	528	532	490	437	509	199
Hazel pollen Bet	304	291	304	244	197	193	2563
Mosquito Cul p	224	231	191	191	192	214	255
Octopus Commo	632	934	275	306	740	1055	2407
Alt a 1 1mg/ml	225	187	135	144	151	142	179
Ara h 2 1mg/ml	32	4	12	19	-8	-14	2
Mouse Lipocalin	302	80	26	61	2395	726	43
Alternaria Enolas	165	207	6	36	88	686	101
Mustard Sin a [	117	118	26	22	254	682	134
Oak pollen Que	304	357	168	163	422	654	277
Apple Bet v 1-like	57	54	20	30	49	56	34
Gly m 4 0.5mg/	90	79	67	78	60	40	77
Rat Lipocalin Ra	295	246	280	292	260	300	255
Blue Mussel My	1025	984	522	644	812	755	5085
Rice Ory s [Seed	187	204	135	133	40	52	165
Squid Lol v	1900	1315	1360	851	1308	1708	1880
Human IgG1 1m	430	455	672	822	609	679	602
Human IgG4 1m	1085	1105	1229	1297	746	1180	1250
Barley Pollen H	522	590	424	492	316	368	662
Candida Cand a	12895	13013	5379	6468	13046	14409	12624
Shrimp Pan b	193	982	338	292	293	273	2345
Snail Hel as	1228	1465	697	744	1150	1739	1478
Human IgM 1m	1038	1313	1134	1309	1106	1082	1454
Cy5 1mg/ml	2491	2863	2749	2849	1832	1952	2264
Barley Seed Ho	486	691	254	334	509	482	830
Corn Pollen Zea	850	771	699	542	570	762	989
Wheat Pollen T	336	332	211	212	168	177	282
American Cockro	1131	1040	485	503	762	897	596
Dog (?) IgA 1mg	4	-5	4	24	-13	-24	-9
Soybean AL3- nat	96	97	122	142	125	175	129
Cockroach, Ame	451	382	384	321	285	318	660
Corn Seed (Flour)	831	195	231	306	21	303	760
Wild Strawberry	167	203	20	36	416	949	-31
Alternaria genuin	492	369	1317	583	432	470	533
Dog (?) IgA 10m	-10	-6	2	3	-21	-8	-16
Soybean AL3- rec	266	396	59	88	153	165	196
Common Sole S	1537	1713	963	1082	786	945	877
Lemon Cit l [Fru	332	141	-21	-28	396	685	4
Cypress genuine	981	985	732	725	729	746	1398
Latex Profilin H	254	229	209	213	419	609	223

German cockroac	2192	2103	1054	1217	1266	1309	1570
German cockroac	3553	4005	275	309	958	1449	7106
Japanese Sardine	367	319	347	426	293	406	525
Lobster Hom g	758	537	1890	1842	966	1082	1872
Cy5	326	360	257	352	333	248	239
Latex genuine ma	179	1475	110	117	69	87	1106
Aspergillus genui	425	446	455	217	162	362	575
German cockroac	6064	5387	447	557	1631	2156	6982
Latex 1 Hevea br	622	652	623	695	528	577	682
Bee venom PLA2	581	535	225	354	627	468	5907
Parvalbumin from	18	13	18	46	4	16	13
Cat albumin Fel	135	48	-3	57	50	69	54
Mugwort genuine	59	80	61	90	122	221	59
Olive pollen genu	213	194	110	127	41	94	171
Horse Albumin	156	141	102	116	179	120	146
Bee venom Melit	180	190	101	133	255	203	296
LTP - Short form	15	23	-6	15	30	17	3
Cow's milk Album	153	145	142	172	155	145	246
Mite Tropomyosi	137	253	264	275	351	156	452
Olive pollen Profi	445	454	549	675	418	675	1476
Bromelain CCD-b	1719	1994	616	918	1165	1935	978
Latex SOD Hev	766	849	595	753	354	474	2449
Act d 2 Act d 2	170	125	113	126	133	109	192
Act c chitinase IV	146	106	92	120	94	87	80
Snail Tropomyosi	601	742	240	318	496	623	334
Sycamore Genuin	725	1179	181	185	136	160	2000
Hen's egg genuin	182	247	413	497	579	776	256
Latex Hevein-like	628	693	4075	4363	1828	2370	3825
Act d 1 Act d 1	74	112	77	108	123	70	55
Act c 5 Act c 5	187	288	343	333	154	205	836
Shrimp Tropomyo	181	289	219	187	157	145	654
Sycamore Genuin	598	765	805	1051	512	657	1216
Wheat Gliadin fra	1568	2073	264	338	217	271	1657
Peanut 7S Vicilin	962	948	946	1020	923	757	3313
Oyster Mushroom	129	155	30	63	509	799	-2
Sheep's milk Ov	354	364	550	515	272	240	652
Kamut Tri tp	244	333	151	238	337	407	366
Corn Seed (Flour)	149	132	-2	146	280	111	166
Latex Enolase H	331	313	468	648	463	427	1744
Hazel pollen Poly	422	484	294	360	826	745	413
Avocado Pers a	255	505	381	548	499	596	432
Tangerine, Mand	349	518	147	283	438	435	664
Durum Wheat T	465	543	410	669	862	1143	684
Oat extract Ave	604	691	359	327	373	409	926
Peanut 7S Vicilin	728	1200	5660	6207	262	264	937
LTP from P. persi	73	52	29	30	17	50	31
Human's milk H	250	197	263	285	401	532	502

Water Buffalo's m	550	353	584	485	179	284	1221
rye extract Sec	13	670	354	-27	2	360	-13
wheat extract T	557	905	-23	371	998	1162	1063
Pru p Hevb5-like	712	112	723	783	491	556	1454
Pru p 3 Pru p 3	96	-4	31	45	45	43	36
Donkey's milk E	224	254	285	340	682	1044	1248
Goat's milk Cap	605	692	653	664	383	393	1508
Flax Lin us	498	517	770	633	1200	1661	260
Camel's milk Ca	737	851	739	727	693	497	660
Pru p 7 Pru p 7	133	144	194	208	167	126	137
Papain Car p 1	252	315	272	504	262	335	423
Parietaria LTP P	1630	1419	1322	1363	206	211	956
Act d 5 Act d 5	186	218	385	413	118	118	982
Rye Grass pollen	736	813	356	422	336	369	697
Salsola PME Sal	856	834	343	389	459	473	3266
HRP as CCD-bear	154	125	134	202	152	126	385
Hen's egg genuin	225	340	129	132	344	317	120
Act d 11 Act d 1	60	71	85	103	113	71	88
Act d 10 Act d 1	151	146	60	657	60	251	55
Palm Profilin Ph	398	458	487	529	636	489	677
Shrimp Tropomyo	467	476	274	383	288	374	767
Cow's milk Lactof	213	129	227	217	656	174	79
Latex Hevein He	434	313	1396	2423	2234	2064	3117
Act c 11 Act c 1	62	89	69	487	78	76	40
Potato Sola t	1391	1227	827	1076	1041	1221	1505
Cy5	260	154	235	259	275	252	245
Cy5	113	63	242	185	203	196	172
Latex genuine ma	95	64	142	145	133	147	265
Latex Patatin H	301	308	376	294	278	251	342
Act c 10 Act c 1	63	85	0	56	69	76	70
Spinach Spi o	252	307	82	218	202	249	498
Sycamore Profilin	6171	7405	288	1145	299	350	300
Cy5	209	263	271	314	204	329	215
Latex genuine ma	1517	1811	1578	1563	1530	1744	4950
Horse myoglobin	273	351	204	255	269	279	220
Cow's milk Bos	281	330	296	307	303	233	1031
Human's colostru	199	297	377	448	278	375	217
Buckwheat Fag	1010	43	-29	224	566	699	505
Rice Ory s [Seed	235	236	274	370	207	198	333
Trypsin inhibitor	63	111	202	109	151	140	163
Potato Sola t 1	1030	1148	819	1012	644	620	5879
Cow's milk Bos	530	719	460	542	268	315	1313
Cow's milk Bos	251	327	344	289	266	284	919
Blood Amaranth	274	290	209	276	478	544	379
Carob Cer si [Se	694	839	353	433	936	830	595
Cow's blood carb	835	938	544	676	374	309	3364

Parvalbumin from	62	97	17	87	37	48	31
Cow's milk Bos	734	865	638	548	357	371	1299
Cow's milk Bos	814	464	684	728	301	368	1372
Camel's milk whe	288	399	357	539	323	318	598
Alpaca's milk Vi	967	731	902	1077	978	887	1074
Timothy pollen	723	653	379	624	118	138	679
Parvalbumin from	505	181	378	285	148	129	119
Cow's milk Bos	670	1305	786	947	360	415	1414
Cow's milk Bos	1049	1027	683	638	239	280	1064
Camel's milk Ca	471	761	905	655	464	506	443
Dog's Albumin C	171	175	129	182	136	108	173
Parietaria Profilin	406	563	673	855	133	170	743
Tomato pulp So	663	432	193	245	230	258	571
Empty	-26	48	44	27	34	18	17
pistachio extract	1638	1678	2005	2005	1532	1488	2914
Asparagus Aspa	294	284	193	194	263	276	259
Celery stalk Api	303	327	-4	237	233	246	255
Bee venom Api	346	324	310	174	350	419	843
Tomato seed S	199	315	222	268	490	660	302
peas extract Pis	140	147	111	140	77	147	86
pine kernel extra	486	481	494	488	431	509	648
Common Beet B	380	394	253	216	293	297	561
Garlic All s [tub	201	205	142	183	121	161	493
European Hornet	284	292	168	162	139	157	181
Cow's milk alpha-	113	137	132	165	112	110	140
banana extract	505	604	84	79	358	476	75
almond extract	1884	1943	1074	1059	1212	1404	2075
Peanut extract	140	212	120	147	119	127	207
Hazelnut Europen	310	307	185	203	314	155	410
Hen's egg genuin	198	172	225	286	190	159	221
Cow's milk beta-L	876	421	382	466	201	373	986
coconut flesh ext	216	275	210	182	172	373	758
carrot extract D	86	67	40	65	26	39	56
Horse Mare's mil	289	291	236	251	297	340	538
Hazel nut extract	169	233	230	273	302	235	233
Hen's egg genuin	288	313	343	277	234	273	284
broad bean extra	236	243	205	238	223	245	230
Cow (beef extrac	420	410	487	484	322	335	451
Cu06 Cu06	324	303	245	253	242	300	445
Helmeted Guinea	260	290	215	248	135	507	184
Helmeted Guinea	1242	912	1512	1654	890	1033	908
sesame Ses i [Se	461	85	175	180	103	146	133
fennel Greek F	607	663	463	402	859	1090	545
CO23 CO23	1002	1051	751	684	928	896	1031
Cu09 Cu09	409	491	487	172	356	302	362
Goose Egg yolk e	611	610	661	731	465	633	553
Duck Egg white e	394	500	1249	1417	362	355	190

pork Sus s [Mea	125	139	90	99	103	105	41
red pepper extra	125	163	10	28	139	193	29
cuN cuN	248	479	780	679	609	585	1976
Cu03 Cu03	631	642	667	540	513	509	1361
Pheasant egg wh	344	279	281	385	340	305	465
Pheasant egg yol	415	403	460	503	275	368	361
wheat extract T	298	222	209	162	115	171	269
Oat extract Ave	421	475	256	215	323	461	362
cu07 cu07	538	358	-10	488	355	466	628
CO110 CO110	67	48	67	58	117	119	99
Peafowl egg yolk	139	180	148	181	150	212	135
Quail egg white e	127	288	244	265	526	213	213
soy bean extract	1586	1447	1476	1494	1313	1321	2464
Empty	23	115	59	397	45	113	113
pear extract Py	72	21	32	52	88	54	564
Cashew extract	1239	1193	1054	1175	1154	1139	733
Onion All c [tub	646	567	571	834	459	576	723
Green kiwi (pulp	98	233	70	76	72	46	93
Gold kiwi (pulp +	159	112	107	115	142	162	132
Apricot Pru ar [	363	391	269	315	230	275	315
harriot bean extr	514	501	446	448	352	470	1009
cod extract Gad	141	119	106	117	106	103	131
Leek All p [tube	284	374	355	422	235	311	482
Lentil Len c	194	204	215	250	224	188	293
Empty	111	95	121	122	179	116	117
Empty	-8	-27	70	45	36	47	32
basil extract Oc	174	177	180	108	116	112	142
lamb extract Ov	358	398	509	653	446	452	670
Hen Egg white ex	368	365	413	414	324	138	354
Hen Egg yolk extr	261	81	255	261	278	242	213
Cow's milk Casein	548	590	363	366	187	224	280
Grape Vit v [Fru	268	344	69	60	708	1312	123
Brazil nut extract	1240	1065	1103	1220	1178	1174	1863
yeast extract Sa	269	308	77	101	102	97	127
Lupine Lup a [S	795	874	-16	682	1156	1351	517
Goose Egg white	319	481	1271	1850	367	525	1111
Hazelnut 11S Glo	365	402	401	503	525	461	891
turkey meat extr	483	476	586	626	300	406	503
AgO+ AgO+	701	625	570	636	633	643	764
cu04 cu04	112	62	76	79	104	55	79
turkey egg white	490	407	504	498	419	408	508
turkey egg yolk e	742	651	743	743	598	572	591
Pecan extract sup	114	136	130	124	109	147	137
rye extract Sec	348	385	332	350	303	388	416
Cu13 Cu13	707	720	863	923	620	699	906
Cu08 Cu08	629	761	468	585	371	413	1131
Duck Egg yolk ext	150	548	128	195	126	135	132

Peafowl egg whit	546	796	819	931	558	593	908
cocomilk extract	1988	2099	1250	1312	695	683	1325
salmon Sal s	2867	249	2083	412	2361	181	1720
SiMVM SiMVM	3050	396	3024	700	2527	254	464
CuX CuX	2758	1346	3129	1516	788	1118	1984
Ostrich egg white	2242	296	2241	714	239	240	412
Ostrich egg yolk	370	312	1853	1800	124	154	417
smoked haddock	286	1836	1565	1754	164	201	89
parsley extract	1867	1955	1515	1779	107	160	92
Cu04 Cu04	631	2389	711	2262	1160	1166	1446
Co112 Co112	270	2415	564	1012	242	246	128
Quail egg yolk ex	940	1368	1403	3015	690	999	830
Anisakis (mediter	533	2861	322	2300	293	2295	644
sun flower seed e	532	2843	595	1689	518	2290	1077
Bipolaris sorokin	851	1089	46	70	700	503	1328
Aspergillus nidu	852	1022	577	614	750	772	2203
Chaetomium glo	833	1024	421	532	968	937	3924
Epicoccum nigru	564	743	255	244	1151	1156	1324
Aspergillus vers	463	686	372	310	562	420	2266
Cladosporium h	902	1272	245	383	818	684	2732
Drechslera spici	2330	2323	534	369	1010	765	7306
Aspergillus flavu	462	513	366	333	660	587	1579
Penicillium digit	868	1253	374	360	1440	1123	4360
Setosphaeria ro	2010	2274	302	352	1016	773	5724
Penicillium expa	1182	1210	430	468	1290	1080	4332
Fusarium solani	238	267	110	56	811	269	3585
Geotrichum can	3457	3675	4813	3460	385	1287	1718
Mucor circinello	984	1204	265	234	427	1290	1342
Kineosporia rhiz	1541	1032	1592	947	293	539	1203
Acinetobacter g	3757	2813	1333	919	4031	4466	1108
Aeromicrobium	256	178	325	376	10	469	823
Erwinia rhapont	5840	5252	1258	1100	1880	2273	1107
Saccharopolysp	1633	1721	1546	1751	1070	1253	881
Eurotium amste	6155	4719	2395	2106	896	1019	3945
Microbacterium	951	1662	330	640	762	1173	1060
Rhodococcus kr	6655	5990	2168	2154	1011	1376	4245
Streptomyces a	2519	1924	1805	1500	1456	1277	4193
Thermoactinom	24105	19565	1728	1633	538	1318	1018

AL22	AL30	AL30	AL35	AL35
453	561	432	255	254
21	64	72	22	35
265	439	472	275	324
1026	573	551	3232	3248
702	946	942	3007	3641
466	861	840	346	314
12387	5196	5136	5802	5554

272	428	406	526	587
749	701	827	1773	2131
11	34	52	65	18
4082	1204	1353	4033	4079
196	288	292	189	123
377	481	565	240	254
211	241	201	163	187
174	252	245	138	170
771	839	846	591	588
700	736	850	500	482
424	453	287	585	543
722	1315	1686	1157	783
-24	23	31	28	13
84	68	85	45	30
340	513	580	318	339
338	224	221	134	116
1404	767	855	415	415
222	408	356	592	436
-22	121	35	-5	-2
0	532	40	82	66
624	1072	1023	1598	1842
570	904	992	368	296
545	203	230	149	172
2266	5987	7803	16239	17996
-1	64	29	10	25
-38	28	-5	18	30
139	514	477	76	119
543	800	878	317	340
257	331	355	177	135
314	644	641	255	299
2113	1738	1637	2883	2410
1677	1682	1638	1056	1009
9106	5317	4678	4379	3524
534	1250	1033	512	452
-7	36	57	-1	6
272	229	295	191	234
653	826	862	128	183
223	414	387	137	140
616	1139	1025	496	578
407	534	545	316	360
340	344	357	245	264
390	450	398	292	410
160	179	181	121	130
324	514	1148	231	186
79	83	284	211	103
326	808	656	277	324
453	465	242	274	310

653	1191	1334	478	298
161	147	264	861	950
540	564	644	342	370
2730	677	742	787	651
457	570	514	318	325
2130	1509	1347	1675	1691
251	255	154	145	216
207	302	388	231	111
-1	75	72	48	28
11605	3696	3544	3964	3469
2101	1039	1142	3264	3168
367	366	465	219	224
910	1323	1396	1443	2070
103	89	85	210	194
517	401	533	1133	905
2756	1457	1616	3520	3539
1409	2129	1632	6221	6007
79	294	294	57	89
483	364	451	401	320
52	62	101	8	90
143	190	230	105	87
229	352	262	233	182
202	221	268	145	140
3082	1571	1605	1000	1154
31	32	-13	5	24
150	100	98	43	64
296	254	360	80	98
790	1495	1502	696	850
202	408	505	117	124
1061	572	691	597	606
430	828	414	261	304
462	822	775	252	168
795	619	543	256	275
971	1832	1676	1024	756
512	371	701	291	336
15	553	10	11	10
343	511	605	296	303
4464	1670	2096	839	1049
169	129	158	211	222
30	71	69	7	96
481	474	438	245	240
44	-15	67	36	28
1891	1672	1515	523	337
1703	937	900	1103	1056
449	423	339	401	378
1085	760	809	362	231
603	647	750	453	446

249	466	370	176	156
265	203	276	82	79
908	544	560	384	208
15	7	54	-7	480
1329	889	897	700	840
2081	1335	1265	407	519
445	315	371	146	140
150	53	-7	218	218
75	-52	-76	203	154
669	631	502	358	420
533	445	473	196	167
3533	3185	2913	2425	2509
287	143	241	1490	1409
449	96	198	345	314
1477	697	675	3268	4131
956	857	845	911	902
2072	1942	1910	1692	1684
3286	3815	3989	2579	2521
1010	1183	1057	4995	4508
333	3751	751	921	1035
1185	647	552	471	369
135	149	168	68	71
8943	8043	7607	6096	6471
1297	1015	1156	1185	1096
57	-123	-67	77	82
1968	1245	1270	3743	3475
946	924	773	485	472
-23	-35	10	-6	-6
547	617	548	383	370
-18	31	26	26	6
284	363	270	308	340
461	662	725	450	549
428	1323	1095	294	233
957	578	548	208	229
736	368	334	37	59
88	271	256	1091	1131
-2	-3	4434	-10	-5
368	490	1158	767	523
1009	678	753	275	397
965	983	986	307	301
1009	3222	3335	95	106
46	145	98	45	55
474	663	642	374	355
1968	325	346	200	181
204	281	240	242	203

2642	738	809	1395	1533
192	305	268	100	109
-8	50	92	191	156
52	63	118	11	60
147	142	156	-14	327
182	-6	34	213	189
278	567	600	320	322
35	71	69	0	49
50	11	82	65	29
276	438	402	170	207
4533	985	744	1863	2356
197	116	137	95	76
2913	1421	1427	1149	1170
594	541	546	318	412
1242	1300	1042	1010	928
528	928	1163	1112	1178
13584	9221	10476	5047	4769
465	709	218	904	838
1350	1099	1237	2126	1892
1525	1600	1715	1090	1140
2254	1755	2717	2094	1603
881	832	894	352	358
1015	2352	2377	1181	1139
408	751	691	195	286
664	883	551	453	433
3	29	18	-8	-2
138	288	228	116	115
986	598	556	2618	2854
466	402	695	627	544
49	-262	-282	388	402
613	861	804	373	351
-15	37	53	17	-12

233	314	368	209	182
1340	1243	1400	458	513
20	-54	-52	86	83
1393	974	968	702	693
267	258	250	129	181
1252	1018	852	927	772
7277	8119	6891	781	777
364	445	454	339	310
1952	953	1057	904	846
226	377	425	242	264

659 258 334 103 148

660 415 425 380 461

7241	9268	8267	1761	1711

500	897	836	454	407
5588	3142	1961	5229	4364
4	99	257	16	9
63	121	86	60	95
70	200	427	72	397
166	1472	290	85	90
173	409	361	124	100
253	112	125	123	121
0	64	100	13	2
232	370	383	90	110
485	419	404	256	108
1613	627	481	1326	1424
1098	2708	2680	2286	1961
2234	1953	1577	326	434
212	168	233	117	119
90	250	202	79	85
312	461	441	184	199
2016	297	299	167	168
489	417	286	200	167
3966	4300	3774	708	771
76	118	632	63	62
1171	1023	963	276	307
376	252	186	203	185
1390	5345	5501	891	823
2164	3588	2781	598	800
3368	1237	1144	713	887
650	49	26	8	5

698	1568	1285	233	193
365	405	348	356	362
247	305	286	215	182
2213	709	622	493	375
481	761	737	378	423
465	761	714	1195	1298
675	593	481	277	344
788	471	454	540	769
986	644	678	323	365
1329	1624	1290	344	305
35	305	270	56	40
559	494	435	225	204
833	1192	937	458	278
-17	-25	1	-24	- 11
1352	1126	991	990	989
1502	993	748	2003	1789
60	401	459	-16	11
926	436	542	361	286
1986	2241	2013	133	295
204	781	680	2749	2588
608	778	748	433	458
111	250	210	88	106
411	840	831	317	380
1107	1171	981	2095	2363
1003	1514	963	300	295
673	2023	1891	706	786
3274	1839	1633	1901	1726
389	1038	877	172	202
558	368	245	93	113
77	125	134	81	58
54	266	224	34	47
828	487	512	238	227
857	387	423	346	404
56	257	241	170	53
2377	3256	2653	673	762
34	67	84	63	67
1113	3469	3231	2368	2037
170	447	372	243	252
171	308	325	186	144
221	232	320	164	166
270	440	387	224	173
41	185	226	57	59
574	359	349	131	189
295	392	385	172	195
229	382	397	247	183
5888	2055	2328	983	1145

220	373	394	281	229
796	615	505	255	246
209	324	337	302	289
579	683	597	925	809
345	517	455	161	128
152	166	159	99	107
5415	6230	6117	888	965
1312	933	933	336	295
866	618	824	307	247
509	632	529	260	277
645	698	706	1517	1701
3876	1157	1071	814	865
371	302	277	34	56
807	1395	1261	494	378
901	1388	1225	523	465
473	851	757	587	383
1312	2112	1685	843	594
762	203	164	261	190
134	1450	1068	81	66
1425	1424	1306	503	536
1101	1073	978	390	412
593	1232	923	532	521
139	224	216	109	132
1170	738	818	241	217
303	426	497	343	304
-5	99	56	13	14
2756	3195	3660	1694	1864
228	438	404	223	192
298	247	322	248	224
758	474	363	925	957
261	302	260	185	178
56	483	470	134	138
645	852	810	391	422
623	541	557	180	205
569	377	377	188	175
195	386	257	194	190
121	1133	859	92	119
53	106	132	1022	972
2376	3099	3332	2091	2087
360	178	214	155	140
193	296	274	214	183
267	228	294	112	140
1071	418	460	246	246
783	444	404	150	180
50	110	94	41	51
692	653	421	193	269
263	709	637	174	173

274	434	373	419	678
211	425	505	279	324
456	482	494	302	297
389	365	454	240	231
175	286	213	307	246
957	919	834	694	735
233	285	330	155	159
581	832	780	1062	1099
917	695	497	417	546
519	610	656	233	202
568	513	480	448	497
179	274	324	137	141
58	145	135	89	109
25	76	26	13	47
1721	1670	1433	411	459
1253	1095	1178	422	436
644	335	378	271	318
241	359	363	227	242
279	242	244	166	162
293	710	344	263	277
674	872	1027	346	288
103	119	223	58	47
141	141	193	131	121
193	248	321	180	171
2297	2000	1977	1143	1195
104	172	168	304	254
22	121	41	50	56
1164	1552	1725	881	991
669	901	859	649	626
153	289	94	6	38
98	261	196	93	101
333	198	206	985	862
1070	633	575	976	976
136	101	144	73	83
461	540	502	297	328
380	434	392	171	168
128	300	257	126	105
3	125	102	52	79
128	187	150	145	147
597	657	616	347	361
347	457	371	257	223
221	296	299	200	205
234	465	495	221	223
161	39	68	203	206
1820	2806	2912	1168	988
147	309	250	142	153
688	1176	1198	2117	1902

1239	224	489	722	776
1120	2553	2373	442	423
516	547	548	333	353
756	615	521	249	292
39	226	75	32	38
427	531	498	337	366
624	612	542	1205	1297
132	245	209	130	210
390	488	562	304	291
1065	848	1091	518	488
1193	736	908	364	388
112	199	186	86	107
913	712	799	502	531
1886	2019	2058	708	915
143	3119	346	124	105
408	3392	647	208	266
2658	1532	1903	896	574
562	309	345	256	285
371	437	519	165	201
101	240	223	160	98
178	299	298	149	100
1791	1445	1498	405	451
357	378	506	68	46
925	808	903	694	833
731	1064	1012	2484	2097
2112	811	2173	570	583
1081	625	307	493	1636
1849	1901	1441	1078	1762
3293	2201	1663	2018	2621
1382	2406	1756	871	1353
2328	1199	1521	654	1473
1943	2123	1488	1577	2983
9750	1137	1437	1018	1936
1590	1237	1089	980	1591
4046	2948	2960	1435	3422
4716	1057	1254	927	2222
3502	1623	1384	1436	2957
2100	812	-98	41	965
1759	649	557	1269	1064
1871	690	281	1181	1304
1543	495	378	469	502
1238	7670	7605	1253	910
849	471	440	85	399
1240	666	782	404	591
878	748	51	641	539
4071	1281	1611	2218	3675

587	1339	1000	513	465
4929	1530	1450	4000	3913
4084	2032	2141	1870	1136
1205	561	558	510	530

Control group

Name	GRACIE	GRACIE	JOSEPHINE	JOSEPHINE	NUGGET	NUGGET	REESIE
Plantain english,	386	363	255	228	267	280	380
Cat Epithelia Fe	115	68	9	1	23	34	89
Beech American	362	312	224	212	308	224	353
Helminthosporiu	645	649	396	374	1570	1364	440
Moth Heterocera	1277	634	442	306	3672	3814	1230
Orchard Dactylis	527	416	388	296	492	462	509
Dock Yellow Rum	1735	1572	672	720	3038	3781	2133
ELM American Ul	358	263	198	234	154	175	246
Malassezia Pachy	481	579	297	350	508	464	549
Flea Ctenocephal	154	50	8	-7	33	24	377
Penicilium notatu	957	1295	781	1048	1019	1460	378
Dandelion Taraxa	196	186	299	321	243	320	294
Bean castor Ric	387	358	198	163	252	292	330
Nettle Urtica Dio	239	248	133	104	214	228	211
Cy5	200	327	144	147	188	208	314
Mouse Epithelia	770	721	538	629	651	618	864
Rat epithelia Ra	552	675	492	517	524	532	665
Alternaria alterna	407	375	114	105	205	175	239
Johson grass Sorg	448	500	994	1000	414	419	679
Birch allergopha	41	20	9	21	10	-18	31
Cat allergopharm	226	207	4	33	37	17	181
Horse Epithelia	463	492	319	392	383	361	555
Sheep's epithelia	287	334	119	98	151	132	227
Fennel dog Eupat	428	439	605	643	875	924	476
Cypress, Arizona	380	381	301	262	929	831	162
Nettle	5	44	15	4	-7	13	59
SFA8 Hel a 2S A	54	22	25	14	37	13	153
Euroglyphus May	998	872	567	550	846	744	1492
Timothy phleum	391	337	418	354	237	294	534
Ragweed, Weste	212	217	276	331	251	189	153
Tyrophagus putre	7790	6382	849	834	14688	12960	8347
Dermatophagoid	50	28	19	22	16	33	81
Dermatophagoid	4	-23	-25	58	22	4	4
IgE Neat	818	660	43	35	115	117	110
Fescue meadow,	394	299	318	301	379	376	498
Mugwort, Comm	270	262	171	150	256	251	322
Grass Smooth Bro	363	276	411	413	851	562	554
Der f 1 Der f 1	980	793	1295	1272	2968	2620	751
Der p 2 Der p 2	1333	1339	1355	1486	1523	1461	918

Bermuda Cynodo	1244	1140	626	806	2647	3391	1879
Rye, Perennial Lo	398	375	432	444	321	342	543
empty	39	29	30	2	19	9	30
Poplar White Pop	272	224	188	189	249	243	343
Der f 2 Der f 2	312	374	127	204	169	136	211
Der p 1 Der p 1	330	344	133	167	185	192	292
Red top grass Agr	421	460	457	414	348	412	573
Hazelnut America	378	516	373	493	319	372	551
Culicoides Culico	398	360	158	157	253	221	360
Maple soft Acer s	503	445	283	289	415	406	596
Eucalyptus Globu	164	195	131	161	206	185	233
Hazelnut America	174	250	252	242	233	219	348
wool sheep ovis a	284	126	161	223	129	111	61
Cow Epitelia catt	1165	1104	255	290	281	280	602
Alder european A	441	438	297	362	370	299	587
Juneky blue Poa p	578	572	417	457	465	442	700
Cedar Red Junipe	330	346	324	351	1071	988	314
Arroyo Willow S	495	463	348	340	368	317	594
Rhizopus nigrican	893	805	822	725	460	411	379
Dog Epithelia C	489	505	288	328	367	378	493
Lamb quarter Ch	1199	1303	854	756	1558	1406	748
Pellitory, wall Par	255	199	131	120	153	232	271
Cy5	359	276	295	218	220	241	355
empty	370	37	2	-4	-6	131	69
Aspergilus niger	520	665	1852	1754	1576	1211	1338
Aureobasidium P	1155	658	581	436	1980	4943	638
Poplar White Pop	211	248	192	195	506	526	362
Lepidoglyphus De	1492	1683	882	975	2169	2805	1947
Acarus Siro Acaru	77	110	22	25	64	84	148
Cladosporium he	190	160	84	92	90	115	219
Aspergillus fumig	1173	1302	1351	1308	1381	1455	988
Blomia Tropicalis	1705	1715	851	845	2612	2595	3101
IgE half dilution	440	377	6	68	66	102	124
ragweed, Short A	660	479	460	436	462	466	477
Ber e 1 Ber e 1	230	203	24	17	101	176	142
Act c 1	186	229	119	94	156	110	138
Birch European B	94	329	235	248	170	255	463
Sycamore platanu	149	257	119	124	258	169	234
Rusian Thistle Sal	707	627	361	379	1076	1203	651
Ash allergophar	25	46	-31	15	-1	1	23
soy bean lectin	61	46	77	68	57	51	143
Soya 11S (Claire)	206	191	157	86	71	86	184
Velvetgrass Holcu	595	581	735	695	424	551	607
Oak English Quer	185	182	81	79	126	120	227
Cockroach, Germ	887	987	451	426	575	451	641
Rye Cultivated se	402	359	225	194	315	321	463
Phl p 1 Phl p 1.0	592	296	260	218	298	214	409

Phl p 12 Phl p 1	491	392	1456	947	719	525	389
Sweet Vernal gra	768	555	361	515	533	598	789
Olive Olea europ	648	644	396	352	543	506	551
IgE one eight	37	47	0	68	247	21	37
Cy5	457	417	270	280	346	333	587
Phl p 6 Phl p 6.0	1895	1972	4019	4118	1968	1352	2878
Phl p 2 Phl p 2.0	117	99	127	106	189	151	611
Sorrel red Rumex	56	135	35	35	71	182	72
Margerite (Ox-ey	285	317	415	396	287	276	410
IgE one quarter	102	91	17	-7	45	24	39
Mite genuine alle	898	819	559	445	352	392	757
Lobster Norway	947	805	773	947	1162	1379	1143
Mosquito Aed c	342	453	259	264	325	294	562
Bet v 1a Bet v 1	654	648	382	368	600	465	753
Bet v 2 Bet v 2.0	464	437	1648	1515	510	438	592
German cockroac	240	248	103	122	101	181	136
Storage mite Eu	167	155	52	87	98	132	116
Melon Cuc m [P	359	292	77	20	177	214	17
Monk fish (angler	512	829	7	11	7	11	85
Par j 2 Par j 2	395	384	495	529	327	298	709
rBer e 1 1mg/m	118	123	291	348	196	193	1485
Dog's Lipocalin	231	216	189	62	225	295	80
Apple Mal d [Fr	8	-54	104	165	474	391	-133
Orange Cit s [Fr	-90	-100	11	65	461	343	-166
Rabbit Dander O	588	576	295	318	463	444	110
Human albumin	400	246	173	175	177	182	320
Human IgG 1mg	2927	2720	2479	2431	1998	2495	2555
Ananas (Pineappl	346	416	158	236	1292	1194	22
Artichoke Cyn s	106	135	180	293	613	591	131
Oyster Common	692	720	311	357	753	766	207
Parietaria Par j	753	836	573	445	670	972	540
Cy3 1mg/ml	2149	2245	2067	2051	2058	2181	3237
Human IgA 1mg	2740	2491	2314	2217	2531	2450	2483
Anisakis (Oceans)	574	425	355	540	829	1201	1148
Chestnut Seed C	596	509	685	761	1722	1459	374
Sunflower Pollen	482	395	1031	927	724	691	447
Tunafish Thu a	133	123	50	36	78	57	149
DogIgG 10mg/m	6979	5722	6774	6694	5543	7267	6797
DogIgG 1mg/m	1555	1354	1408	1350	1353	1165	1432
celery extract A	-122	-103	-31	45	320	292	5
Cockroach orient	1195	1150	828	837	1308	1489	1261
Tiger Nut (Chufa)	453	573	480	490	666	751	502
Trout Onc m [M	15	0	213	6	-19	17	17
Dog IgM 1mg/m	482	365	328	352	406	425	578
Dog (?) IgE 10u	180	33	18	-15	3	-20	15
Chestnut Pollen	303	189	369	266	459	553	341
Hamster Epithelia	658	600	620	602	672	598	777

Anisakis genuine	439	355	757	690	352	331	489
Bermuda grass Pr	436	361	1033	869	553	490	451
Peanut 7S Vicilin	179	268	159	141	102	78	152
Peanut 2S Album	164	149	179	181	81	128	267
Empty	-16	6	-20	15	1	29	14
Hop pollen Hum	780	919	446	454	1181	864	529
Anisakis Tropomy	557	557	873	867	372	323	477
Annual Mercury P	664	556	947	992	550	549	549
Ragweed genuine	211	277	53	63	175	167	174
Peanut 2S Album	156	177	-3	10	36	14	455
Guinea pig Epithe	431	260	415	389	432	725	570
Hazel pollen Bet	305	281	202	175	192	190	357
Mosquito Cul p	293	341	154	156	223	226	359
Octopus Commo	1002	748	676	847	1081	1279	1246
Alt a 1 1mg/ml	167	173	66	77	98	113	224
Ara h 2 1mg/ml	0	41	11	190	180	21	19
Mouse Lipocalin	46	76	1396	110	134	90	91
Alternaria Enolas	113	41	205	160	21	18	149
Mustard Sin a [	-24	-67	84	92	360	260	-79
Oak pollen Que	278	209	256	226	608	482	337
Apple Bet v 1-like	22	53	10	16	22	28	75
Gly m 4 0.5mg/	91	103	36	13	552	495	93
Rat Lipocalin Ra	462	354	175	197	255	230	239
Blue Mussel My	1432	1385	1101	1102	1299	1307	1278
Rice Ory s [Seed	64	80	125	135	148	128	16
Squid Lol v	1527	1078	702	752	1184	1492	856
Human IgG1 1m	502	379	551	454	456	444	494
Human IgG4 1m	1086	950	1076	952	932	937	956
Barley Pollen H	615	654	795	1073	918	1825	638
Candida Cand a	10107	9829	10273	11259	4697	4493	6531
Shrimp Pan b	979	502	207	1164	499	533	427
Snail Hel as	1361	1635	1371	1278	2482	1555	1198
Human IgM 1m	1471	1072	1092	1127	965	981	1030
Cy5 1mg/ml	2351	1896	1856	1857	2380	2368	3028
Barley Seed Ho	694	686	220	362	497	492	491
Corn Pollen Zea	993	647	657	693	1694	1649	586
Wheat Pollen T	264	212	189	187	346	314	387
American Cockro	944	531	814	629	1861	1734	778
Dog (?) IgA 1mg	33	29	4	-1	25	9	90
Soybean AL3- nat	172	156	330	159	216	209	209
Cockroach, Ame	674	649	241	279	397	486	733
Corn Seed (Flour)	570	405	200	643	879	807	193
Wild Strawberry	-347	-450	-23	16	460	262	-439
Alternaria genuin	597	536	439	390	580	445	544
Dog (?) IgA 10m	-9	10	-25	-3	-9	8	18
Soybean AL3- rec	85	95	233	242	133	147	354

Common Sole S	893	682	419	567	983	609	1001
Lemon Cit l [Fru	-102	-127	20	37	320	433	-105
Cypress genuine	1790	1375	750	702	4345	5656	740
Latex Profilin H	208	219	350	297	317	327	246
German cockroac	743	893	991	852	905	802	769
German cockroac	872	1111	3322	2293	1511	1278	932
Japanese Sardine	417	313	343	344	519	461	457
Lobster Hom g	755	699	935	758	1315	1351	2078
Cy5	315	316	237	257	232	261	462
Latex genuine ma	398	266	131	83	96	133	173
Aspergillus genui	339	205	206	213	193	194	330
German cockroac	1524	1482	4623	4278	1973	1711	1391
Latex 1 Hevea br	883	710	530	509	615	374	698
Bee venom PLA2	2586	2779	714	959	2156	2230	399
Parvalbumin from	49	87	39	9	34	28	42
Cat albumin Fel	106	105	39	48	59	64	132
Mugwort genuine	103	99	147	174	128	153	86
Olive pollen genu	122	107	81	117	135	121	115
Horse Albumin	233	205	71	75	79	76	145
Bee venom Melit	271	214	87	88	100	122	143
LTP - Short form	2	84	-1	16	21	26	56
Cow's milk Album	290	334	203	221	161	148	246
Mite Tropomyosi	278	216	288	285	140	114	196
Olive pollen Profi	446	271	603	682	428	344	327
Bromelain CCD-b	1193	918	1198	900	1152	1464	845
Latex SOD Hev	1057	889	597	383	524	625	512
Act d 2 Act d 2	111	50	95	70	88	93	175
Act c chitinase IV	170	176	86	90	102	89	178
Snail Tropomyosi	722	545	392	293	931	947	434
Sycamore Genuin	317	240	127	139	183	213	231
Hen's egg genuin	715	684	211	210	460	470	190
Latex Hevein-like	521	488	840	945	626	590	1237
Act d 1 Act d 1	133	147	81	76	88	89	133
Act c 5 Act c 5	175	166	93	96	141	109	129
Shrimp Tropomyo	550	396	323	325	156	130	297
Sycamore Genuin	1235	1341	796	603	801	756	732
Wheat Gliadin fra	67	49	6	15	229	112	260
Peanut 7S Vicilin	734	699	602	581	706	952	420
Oyster Mushroom	67	111	-18	6	34	10	36
Sheep's milk Ov	576	455	1248	1194	283	311	360
Kamut Tri tp	331	335	140	150	209	273	266
Corn Seed (Flour)	247	262	190	166	185	170	263
Latex Enolase H	545	480	389	684	544	491	875
Hazel pollen Poly	308	305	247	181	366	255	752
Avocado Pers a	825	682	378	213	1266	1336	291
Tangerine, Mand	490	410	220	266	645	501	304
Durum Wheat T	639	595	309	295	511	398	676

Oat extract Ave	510	451	319	279	398	308	514
Peanut 7S Vicilin	1071	835	236	170	318	172	398
LTP from P. persi	145	133	-12	1	289	413	86
Human's milk H	251	199	218	183	198	218	351
Water Buffalo's m	613	529	1084	1309	327	387	492
rye extract Sec	489	513	18	-11	-14	-3	25
wheat extract T	760	569	525	562	641	683	673
Pru p Hevb5-like	1537	1357	772	713	1060	992	1114
Pru p 3 Pru p 3	154	150	3	-7	339	340	106
Donkey's milk E	246	303	156	151	217	211	282
Goat's milk Cap	904	765	1291	1326	160	472	694
Flax Lin us	587	106	-2	25	-24	139	18
Camel's milk Ca	1079	1050	864	703	370	337	423
Pru p 7 Pru p 7	160	199	92	78	181	145	184
Papain Car p 1	587	559	894	368	418	405	184
Parietaria LTP P	1349	1028	481	447	462	425	678
Act d 5 Act d 5	183	175	146	156	179	190	239
Rye Grass pollen	449	428	924	904	459	507	623
Salsola PME Sal	1844	1431	533	505	948	958	739
HRP as CCD-bear	249	283	124	136	155	177	202
Hen's egg genuin	1091	810	542	616	62	83	148
Act d 11 Act d 1	158	153	111	124	92	91	168
Act d 10 Act d 1	294	248	367	278	347	129	696
Palm Profilin Ph	474	443	1340	1070	716	535	329
Shrimp Tropomyo	1099	957	691	716	307	190	394
Cow's milk Lactof	100	82	105	55	57	25	163
Latex Hevein He	389	355	1101	650	492	594	1824
Act c 11 Act c 1	102	103	48	218	1352	68	157
Potato Sola t	1410	1447	995	955	2160	2293	1232
Cy5	305	300	195	196	206	279	380
Cy5	210	200	187	172	151	164	336
Latex genuine ma	260	313	129	129	88	107	110
Latex Patatin H	418	382	157	204	211	247	160
Act c 10 Act c 1	135	130	156	9	3	13	133
Spinach Spi o	328	256	176	196	197	247	281
Sycamore Profilin	313	295	419	474	305	259	313
Cy5	333	271	223	213	220	217	424
Latex genuine ma	701	637	217	308	900	1128	1020
Horse myoglobin	340	465	348	277	273	286	443
Cow's milk Bos	348	331	309	328	259	261	445
Human's colostru	282	327	340	250	673	681	253
Buckwheat Fag	454	462	468	536	710	664	513
Rice Ory s [Seed	259	281	250	214	184	188	341
Trypsin inhibitor	146	172	129	119	149	159	173
Potato Sola t 1	613	735	679	766	1950	2100	1850
Cow's milk Bos	617	654	487	505	346	355	547
Cow's milk Bos	654	565	331	361	478	406	409

Blood Amaranth	399	305	426	383	420	343	347
Carob Cer si [Se	572	697	610	555	1674	1943	395
Cow's blood carb	2416	3199	385	347	422	292	656
Parvalbumin from	50	55	44	14	33	68	79
Cow's milk Bos	1071	860	565	464	448	528	685
Cow's milk Bos	1039	942	604	357	412	1013	701
Camel's milk whe	806	696	412	349	198	331	420
Alpaca's milk Vi	1788	1342	1626	1346	815	812	751
Timothy pollen	1820	1491	731	797	137	159	265
Parvalbumin from	112	105	89	126	112	95	168
Cow's milk Bos	1209	1158	604	608	602	616	812
Cow's milk Bos	1229	867	508	563	534	635	557
Camel's milk Ca	1182	1121	973	1077	389	455	283
Dog's Albumin C	73	160	121	112	102	113	168
Parietaria Profilin	220	413	557	648	1170	697	290
Tomato pulp So	592	500	331	344	417	402	242
Empty	30	1636	533	13	23	24	64
pistachio extract	2378	2171	1715	1765	1892	2698	2188
Asparagus Aspa	308	289	182	205	147	225	257
Celery stalk Api	322	240	137	138	303	239	174
Bee venom Api	397	352	216	201	339	348	287
Tomato seed S	292	337	112	151	250	230	230
peas extract Pis	121	138	169	212	195	187	243
pine kernel extra	654	705	488	504	630	567	795
Common Beet B	360	415	150	122	208	229	228
Garlic All s [tub	218	236	182	137	163	175	226
European Hornet	166	159	176	175	181	244	279
Cow's milk alpha-	222	183	79	99	101	116	111
banana extract	489	377	149	135	786	828	93
almond extract	2440	2278	1114	1182	1704	1760	1699
Peanut extract	129	134	529	221	190	178	334
Hazelnut Europen	254	233	299	237	377	450	349
Hen's egg genuin	200	202	113	155	194	171	197
Cow's milk beta-L	398	348	162	221	243	205	197
coconut flesh ext	578	613	158	183	203	213	230
carrot extract D	69	78	44	41	55	56	127
Horse Mare's mil	230	236	133	166	206	169	286
Hazel nut extract	314	378	930	111	101	111	307
Hen's egg genuin	308	311	174	203	206	158	254
broad bean extra	359	330	193	214	284	279	244
Cow (beef extrac	410	423	293	315	360	344	440
Cu06 Cu06	304	310	407	409	293	251	523
Helmeted Guinea	167	209	371	328	417	388	260
Helmeted Guinea	572	738	679	669	723	1090	482
sesame Ses i [Se	188	308	503	155	211	193	161
fennel Greek F	670	612	340	412	457	492	516
CO23 CO23	647	586	668	673	845	767	1232

Cu09 Cu09	291	357	281	263	290	303	322
Goose Egg yolk e	439	466	365	419	411	553	489
Duck Egg white e	220	213	75	119	132	131	123
pork Sus s [Mea	126	80	57	72	90	65	108
red pepper extra	46	83	-6	36	85	115	96
cuN cuN	760	644	551	533	558	463	386
Cu03 Cu03	1228	990	603	503	544	439	485
Pheasant egg wh	541	330	240	225	311	343	269
Pheasant egg yol	189	203	149	158	176	200	135
wheat extract T	155	237	147	122	181	130	213
Oat extract Ave	198	200	133	131	194	164	197
cu07 cu07	488	394	410	414	413	376	365
CO110 CO110	45	48	69	83	65	51	69
Peafowl egg yolk	102	148	78	97	137	135	260
Quail egg white e	229	244	158	164	250	225	188
soy bean extract	1564	1696	1255	1350	1599	1509	2263
Empty	120	122	57	88	113	140	95
pear extract Py	40	408	39	36	16	36	63
Cashew extract	1151	1159	1072	1093	994	1083	912
Onion All c [tub	615	680	750	843	599	691	369
Green kiwi (pulp	30	240	3	21	38	33	34
Gold kiwi (pulp +	111	111	139	156	149	114	150
Apricot Pru ar [	567	522	306	358	993	887	187
harriot bean extr	1081	1029	417	404	548	618	1004
cod extract Gad	129	125	85	76	80	91	117
Leek All p [tube	365	383	215	195	272	294	197
Lentil Len c	198	259	106	165	158	170	125
Empty	180	79	92	105	251	174	128
Empty	110	66	-52	10	-13	-3	43
basil extract Oc	182	226	89	104	130	138	226
lamb extract Ov	547	672	404	396	846	448	467
Hen Egg white ex	532	531	274	267	221	298	178
Hen Egg yolk extr	175	202	142	162	190	189	177
Cow's milk Casein	466	787	147	143	193	220	146
Grape Vit v [Fru	12	2	90	123	475	514	63
Brazil nut extract	1484	1429	1249	1435	1548	1638	1554
yeast extract Sa	113	78	192	146	173	168	128
Lupine Lup a [S	969	1057	989	875	2414	2346	530
Goose Egg white	652	809	510	578	353	359	322
Hazelnut 11S Glo	1023	1022	286	362	494	391	439
turkey meat extr	513	473	385	410	435	458	491
AgO+ AgO+	784	713	90	165	6385	135	254
cu04 cu04	21	107	16	28	21	19	59
turkey egg white	377	539	282	318	321	338	313
turkey egg yolk e	431	487	488	488	558	581	522
Pecan extract sup	182	166	223	113	168	177	211
rye extract Sec	425	457	315	302	393	359	463

Cu13 Cu13	763	717	698	572	623	507	569
Cu08 Cu08	582	568	606	658	525	477	683
Duck Egg yolk ext	109	111	91	95	101	94	160
Peafowl egg whit	592	670	467	525	1004	953	726
cocomilk extract	1522	1207	1578	1554	1741	1372	1541
salmon Sal s	2069	187	53	162	376	137	76
SiMVM SiMVM	611	492	317	857	8	401	461
CuX CuX	1557	1379	192	207	649	596	819
Ostrich egg white	285	334	551	535	243	216	322
Ostrich egg yolk	218	251	59	100	152	178	192
smoked haddock	198	123	95	124	127	164	216
parsley extract	207	179	1076	965	129	158	189
Cu04 Cu04	1290	1406	17	4	604	598	642
Co112 Co112	58	83	940	903	254	50	73
Quail egg yolk ex	707	792	468	410	998	1010	801
Anisakis (mediter	377	338	579	615	927	927	987
sun flower seed e	641	655	91	48	1035	928	791
Bipolaris sorokin	211	174	334	355	505	944	864
Aspergillus nidu	1412	1532	602	979	1315	1649	1703
Chaetomium glo	1101	1300	856	960	1471	1613	1847
Epicoccum nigru	706	816	515	678	2139	2119	1860
Aspergillus vers	727	858	589	478	750	1086	1388
Cladosporium h	979	1121	815	1325	936	1385	1975
Drechslera spici	773	1100	661	857	957	968	1574
Aspergillus flavu	565	594	504	1081	1045	1034	1066
Penicillium digit	1462	1416	1579	1882	1825	1972	3445
Setosphaeria ro	749	819	660	882	1062	1337	1467
Penicillium expa	989	1044	1107	1166	1552	1635	1647
Fusarium solani	180	-124	-92	37	40	90	202
Geotrichum can	2478	1760	1956	1806	817	825	402
Mucor circinello	1246	1221	372	191	1575	1316	229
Kineosporia rhiz	1824	881	1808	755	1801	797	456
Acinetobacter g	4647	2330	3526	1960	4490	2771	1897
Aeromicrobium	104	888	236	651	570	632	-22
Erwinia rhapont	3222	2396	5166	5584	2233	1406	2084
Saccharopolysp	1494	1710	1738	2072	2090	1486	854
Eurotium amste	2656	1729	2242	1940	1496	1025	1030
Microbacterium	1194	1948	1035	1548	779	1027	603
Rhodococcus kr	1983	2128	3125	2746	1346	1552	942
Streptomyces a	2799	2412	2002	1684	1500	1974	961
Thermoactinom	9992	6252	2815	1194	1775	1586	701

REESIE	EASY	EASY	WRANGLER	WRANGLER
369	292	283	301	317
64	59	94	106	55
333	231	230	228	275

409	644	617	417	545
646	445	551	886	707
514	341	295	460	495
1977	3022	3162	2558	2125
232	277	255	276	272
623	374	350	400	631
275	200	144	380	650
556	763	869	1457	1348
179	161	98	227	176
357	282	290	340	346
216	190	164	156	179
269	249	211	223	213
666	627	629	734	752
660	548	602	619	577
280	168	209	191	187
686	404	489	753	744
20	55	25	26	27
58	59	75	67	69
496	452	446	444	468
198	150	147	171	175
422	236	295	417	416
215	451	304	251	238
42	22	27	17	17
195	28	55	68	59
1145	478	409	689	635
481	285	332	521	473
161	108	118	164	157
7643	9533	12079	5479	4594
52	37	28	25	81
180	10	-4	22	4
84	219	320	285	256
455	345	398	392	453
202	245	248	223	271
617	350	352	383	937
810	1565	1177	1474	1347
876	984	1068	816	923
1812	2708	2722	1832	2437
618	359	345	601	594
1103	4	9	25	28
338	271	242	351	331
201	118	114	269	287
254	239	250	211	211
506	411	363	574	565
540	354	364	347	398
339	242	247	288	297
626	276	284	348	366

203	160	235	448	152
323	238	203	244	549
124	148	110	354	161
540	543	552	607	583
548	396	386	307	381
605	448	488	780	665
219	716	513	257	356
489	351	417	496	539
346	454	512	477	449
495	409	439	406	407
686	636	722	924	1008
263	145	134	166	173
327	232	297	310	283
72	64	41	187	136
1105	1651	1434	1839	1637
407	-19	444	1169	1175
308	267	273	290	323
1906	1088	1042	975	1032
133	24	61	71	95
227	233	208	308	355
954	874	1179	1307	1293
3175	1287	1588	1189	1333
161	217	236	231	157
529	430	346	163	343
106	35	280	307	38
110	121	171	77	99
379	1670	206	297	355
180	158	160	112	207
671	831	937	774	727
26	29	33	21	37
67	50	77	98	87
199	138	112	131	151

601	463	463	719	609
168	126	136	197	213
618	403	326	471	479
409	338	284	398	414
332	267	252	286	340
449	2141	1923	424	342
597	596	451	773	528
700	478	505	450	421
15	25	46	39	19
651	410	345	412	330
2871	1656	1745	1771	1790
161	113	100	114	130
65	39	44	17	38
364	265	264	307	399
34	83	84	38	26
668	254	264	761	774
1076	1002	942	1191	1187
557	388	325	400	402
763	567	595	543	526
501	3044	2520	367	390
118	119	151	110	97
95	112	106	89	88
22	-23	2	432	-3
33	30	64	35	593
740	305	350	1112	948
1227	54	48	837	1049
83	152	134	118	138
-41	322	166	-71	-12
-63	193	118	-123	-72
170	355	307	38	90
310	279	234	241	402

2639	2790	2915	2131	1942
94	424	424	263	400
118	327	330	85	137
261	188	222	200	293
792	743	750	630	689
2779	3249	2680	2273	3032
2371	1921	1842	1664	2076
1089	1168	1235	679	718
413	435	419	411	683
505	299	311	311	419
103	118	73	126	136
6984	5167	7209	4887	5279
1220	1409	1852	1169	951
-90	119	130	-61	-7
1149	811	777	808	738
462	459	459	592	660
-3	32	33	8	28
819	451	467	296	313
35	8	86	30	39
260	250	329	300	286
796	670	710	729	649
464	327	277	312	345
399	406	367	334	343
126	167	121	175	151
246	149	204	231	238
72	-12	33	14	37
446	527	589	551	628
448	395	303	431	429
560	654	589	526	524
106	180	184	259	235
36	70	27	39	7

327	470	501	459	445
510	297	302	260	294
363	234	197	235	231
1591	325	283	475	612
89	59	42	83	112
280	104	2742	61	53
660	23	1950	1158	738
248	286	469	1171	1230
-26	270	145	-50	34
274	274	287	283	323
54	50	50	25	56
25	48	41	13	20
177	271	266	159	196
1362	684	861	637	717
41	44	65	87	99
1094	642	545	505	510
385	559	542	360	360
957	853	1111	810	774
848	641	822	741	684
6345	8730	7212	6684	6982
922	662	1115	1096	1179
1315	1847	2399	894	890
983	1061	1297	494	535
2996	1996	1941	1504	2110
339	397	384	448	452
621	760	616	921	1266
404	366	265	357	331
692	594	689	691	704
47	-5	34	14	38
213	131	212	232	235
641	381	430	565	472
215	421	383	247	402
-384	91	1	-180	-136
553	493	527	432	371
20	51	5	-1	-1
309	76	80	692	749
688	562	560	838	1061
-68	204	179	-122	-97
636	1593	1392	485	445
274	153	177	165	150
732	694	735	911	718
1005	714	546	1866	2078
474	353	364	668	638
1755	934	810	781	779
434	333	343	327	299
164	137	138	132	168
279	249	275	176	225

1513	1045	995	3123	3567
549	585	626	635	595
405	738	916	442	762
58	-2	56	112	38
155	91	126	69	85
63	110	107	175	214
175	97	70	155	147
133	108	97	127	121
35	81	50	165	178
79	41	32	26	-3
260	202	236	145	188
180	198	172	230	252
359	320	304	481	479
713	1210	984	1066	1185
482	489	456	264	350
208	138	113	98	176
176	118	119	170	112
432	500	351	321	262
203	144	142	213	207
168	145	213	250	220
1730	773	751	1511	1204
100	116	410	81	98
140	276	244	157	120
250	196	139	178	254
737	693	731	767	898
341	119	120	907	847
378	452	476	364	423
3	29	65	43	7
326	358	346	1295	1244
239	158	141	230	222
199	269	283	199	199
768	708	684	641	620
823	158	188	386	449
244	575	906	400	535
154	259	327	221	243
475	312	235	332	433
482	358	402	431	495
465	192	174	249	268
70	76	40	149	120
294	169	187	514	457
399	408	368	1149	905
468	-2	12	18	-2
528	367	455	514	478
1050	463	458	809	918
92	101	135	177	240
259	206	217	411	370
762	553	415	1471	1413

35	297	-14	11	24
418	418	384	408	375
153	120	173	160	149
214	264	311	139	124
593	263	203	737	831
179	451	312	134	111
577	489	515	857	865
748	1257	1318	752	730
174	136	159	205	193
111	809	884	92	55
163	142	68	111	118
342	385	381	261	282
429	320	298	291	392
401	346	382	301	281
131	98	140	121	107
1256	687	626	1067	976
126	104	47	213	45
1088	982	809	1720	1798
321	253	235	312	257
300	250	222	258	286
109	143	104	122	120
160	129	144	119	110
96	80	46	87	123
262	176	147	188	243
395	266	188	259	250
384	329	228	321	319
1176	246	245	955	906
461	364	353	318	325
358	288	307	742	770
157	202	185	278	281
429	275	348	326	330
292	230	244	313	344

212	126	132	121	148
2204	1347	1614	1769	1925
623	423	382	2219	1511
471	437	409	977	1202
304	268	239	380	401
415	767	971	647	932
682	410	353	785	913
75	52	35	100	61
532	444	413	1458	1387
659	603	629	1707	1518
400	362	549	511	537
682	628	778	1124	1120
300	78	82	172	227
169	106	115	179	191
822	508	482	1902	2070
608	347	393	1649	1636
367	417	410	672	725
158	117	123	120	-7
293	254	310	270	325
282	370	359	259	219
35	-7	25	37	17
2087	1537	1601	2198	2201
226	183	239	181	190
158	105	150	86	114
291	171	195	265	299
296	190	232	119	112
247	106	119	117	159
818	471	525	844	883
167	138	126	235	181
309	153	150	166	168
331	178	184	238	235

135	92	142	197	169
118	183	180	112	115
1528	925	891	1763	1826
359	231	278	213	199
344	212	206	294	231
153	137	171	338	273
160	122	145	3826	509
242	190	187	1072	285
98	113	69	25	74
274	202	224	396	396
282	140	107	116	105
251	244	222	205	166
279	261	271	260	235
393	363	346	327	287
401	329	301	247	275
339	314	331	1011	425
426	475	634	613	618
200	146	176	12476	222
608	356	407	5591	710
1144	486	601	1790	869
329	353	351	371	359
529	374	397	449	375
69	110	54	94	90
120	121	80	82	107
-17	9	37	20	78
325	656	702	792	672
497	457	459	507	512
305	184	244	266	202
113	158	165	12672	151
210	151	184	49	178
164	199	214	124	162
435	329	332	10796	14180

97	90	67	36	98
162	107	126	218	366
228	183	174	174	215

2499	1052	1138	1457	1391
98	122	82	66	93
33	53	27	47	37
627	724	760	1127	1086
741	379	423	6038	194
51	33	73	-5	63
159	100	147	162	104
215	323	439	119	202
1073	511	563	607	553
123	135	134	72	127
265	198	157	171	177
124	116	145	86	81
101	93	107	120	78
65	99	53	-48	45
259	96	148	389	124
441	378	436	367	332
210	300	339	53	136
243	181	200	13899	208
152	131	143	5653	128
62	209	352	178	59
2208	1381	1511	1577	1864
106	74	138	4455	172
539	984	1183	250	384
1692	383	439	280	298
649	209	278	199	122
478	384	442	130	426
207	189	164	244	266
81	54	59	68	21
289	246	326	362	276
508	410	539	478	455
235	113	190	350	152
388	312	397	934	391
697	510	640	1447	604
684	477	507	1057	464
4270	120	138	3569	142
1530	594	563	555	622
2193	698	829	2303	2426
147	120	163	97	160
469	297	305	371	325
792	773	851	498	651
329	266	285	158	264
211	112	216	744	261

200	113	154	109	142
160	156	145	10119	132
565	728	740	437	529
65	54	40	-441	845
770	811	882	3197	353
1406	677	718	455	430
1852	570	615	718	501
652	503	1073	1051	598
1428	924	1383	2274	2060
1416	1218	1723	2324	2203
1523	982	1300	2322	1891
1240	886	1486	1726	1414
1417	994	1044	3469	2253
1100	715	981	2269	1917
1014	842	959	1358	1222
4212	1428	2230	3226	2286
1515	880	1566	1844	1503
1225	1137	1745	2290	2062
27	400	114	1704	1002
289	377	297	571	633
325	332	376	284	567
559	309	439	897	925
1553	2869	2587	2083	1676
19	-65	135	152	395
1697	396	596	822	974
725	731	665	642	518
1176	799	975	2575	3006
1031	841	771	1085	356
1099	389	837	1812	1784
821	1049	935	1481	1366
537	707	623	423	505

Appendix E: PLS-DA VIP output

Allergome Name	Genus species	VIP Scores for Y 1	VIP Scores for Y 2	VIP Scores for Y 3
Hev b 11	Bos domesticus	3.02675991	3.81865816	1.60797014
Hev b 6.02	Actinidia deliciosa	2.83170672	3.54372114	1.31276485
rAsp f 8	Rhodococcus kroppenstedtii	2.55710507	3.32269079	1.27680304
Hev b 5.0101	Alternaria alternata	2.63640157	3.31179626	1.09689545
Hel as 7	Euroglyphus maynei	2.14809049	2.70964881	1.09753936
Bet v 2.0101	Culicoides nubeculosus	1.83398979	2.35666626	0.84431984
Hev b 3.0101	Solanum tuberosum	1.83326107	2.29597847	0.96296138
Cul nu 2	Bos domesticus	1.80166433	2.15527893	0.62047375
Bla g 2	Armoracia rusticana	1.64779306	2.12820768	0.75092619
C0145	Bos domesticus	2.83425938	2.01994683	4.11201351
Mer a 1	Fagopyrum esculentum	1.59393926	1.97716495	0.79492952
Bos d LF	Phleum pratense	1.54556631	1.88509187	0.72838178
Muc ci	Parietaria officinalis	1.43866994	1.82343258	0.63756411
Cul o 2	Culicoides obsoletus	1.63876961	1.8129501	0.5248506
The v	Ctenocephalides felis	1.40405546	1.79995744	0.73128915
Eup c	Bos domesticus	1.30979374	1.75302145	0.73648241
Geo c	Aspegillus flavus	1.23368246	1.74591876	0.7331518
Hev b	Culicoides obsoletus	1.34286348	1.69173203	0.59895766
Eur s	Candida albicans	1.32557758	1.69015508	0.71199357
Pru p 3	Leucanthemum vulgare	1.4727471	1.67626241	1.04511526
Rat n [Epithelium]	Aspergillus fumigatus	1.37228958	1.65449104	1.1137642
Blo t	Platanus acerifolia	1.4494602	1.6318998	0.95761978
Cul 01P	Culicoides obsoletus	2.61249726	1.58820356	3.81569472
Der f 1	Carpinus betulus	1.30356951	1.58415415	1.2547587
Per a 7	Culicoides nubeculosus	1.16630203	1.58027656	0.7708249

Fel d [Epithelia]	Glycine max	1.5517931	1.56515701	1.00123987
Cy5	Culicoides nubeculosus	1.26419188	1.56148192	0.50165174
Can f [Epithelium]	Mercurialis annua	1.22915936	1.54516853	0.73011186
Gal d [Egg White]	Culicoides obsoletus	1.35682775	1.54432871	0.88729721
Que r [Pollen]	Helianthus annuus	1.42890712	1.53220564	0.65478524
Der p 2	Corylus avellana	1.21526498	1.52321279	1.19375943
Hel a [Pollen]	Culicoides nubeculosus	1.12626698	1.5082573	0.61719544
Cul n 10.03	Zea mays	1.49037119	1.50450002	1.02336867
Soybean AL3-recombinant 1mg/ml	Culicoides obsoletus	1.49654019	1.4831104	1.06767378
Sim vi	Periplaneta americana	1.1795619	1.47618731	0.92770137
Hel as 7	culicidae	1.19753497	1.47212102	0.53249424
Epi p	Acarus siro	1.37374289	1.45566192	1.02092096
Ulm a	Zea mays	1.45485511	1.45480509	0.97916378
Zea m [Seed]	Culicoides nubeculosus	1.28169212	1.44381482	0.48004773
Art v 1	Corylus avellana	1.07738089	1.43863331	0.7732555
Cor am	Gallus domesticus	1.25097433	1.42480261	0.29901115
Act d 2	Culicoides nubeculosus	1.39316454	1.42357073	0.85991931
Par j 1	Platanus acerifolia	1.33666691	1.4203038	0.57637802
PBS	Culicoides obsoletus	1.34002374	1.40870269	0.74049142
Lup a [Seed]	Culicoides obsoletus	1.33442798	1.40796374	0.67432125
Ole e 2	Mus musculus	1.15003853	1.40634232	0.61897686
Bla o	Culicoides obsoletus	1.09158817	1.39811819	0.90152377
Par j	Culicoides obsoletus	1.24589724	1.37787051	0.59231285
Pen m 1	Acer saccharinum	1.28473777	1.34957937	1.08190841
Sola l LTP_7kD	Apis mellifera	1.16577311	1.34626132	0.60747854
Kin r	Poa pratensis	1.20471283	1.34037522	0.36795526
Cul o 8C11	Bos domesticus	1.21704575	1.33855759	0.34145902
Gal d 4	Culicoides nubeculosus	1.2494067	1.33836611	0.66742486
Ole e [Pollen]	Triticum turgidum ssp. durum	1.27289333	1.33529965	0.6282347

Hel a 2S Albumin	Controls	1.2429888	1.33485809	0.71724112
Ole e 1	Zea mays	1.2151521	1.33149476	0.13444526
Pen e	Blattella germanica	1.11291792	1.32983975	0.99468872
Asp fl	Saccharomyces cerevisiae	1.21013661	1.31154371	0.32566064
Ara h 6	Felis domesticus	1.31323198	1.29759913	0.96848856
C0120	Ovis aries	2.14429432	1.26816106	3.13341348
Per a 7 (0.5mg)	Culicoides	1.0054201	1.25257037	0.6106231
Cul nu 12	Quercus robur	1.00820067	1.23789222	0.38905664
Fag e	Culicoides	1.09273767	1.21303447	0.32774469
Cor a [Seed]	Culicoides obsoletus	1.26842929	1.20748375	1.04627577
Sac c	Culicoides nubeculosus	0.95284042	1.20353847	0.8891573
Cul o 1	Equus caballus	1.12287172	1.19804805	0.43110926
Act c 10	Bos domesticus	1.16719348	1.19718914	0.70547945
Euc g [Pollen]	Phleum pratense	1.10731775	1.19303577	0.5038297
Pla a 8	Culicoides obsoletus	0.90237697	1.18079271	0.8131368
Phl p 1.0102	Populus alba	0.94963859	1.17720949	0.78793236
Cla h	Ceratonia siliqua	0.85533978	1.17660751	0.70593535
Cul o 6 F10	Culicoides nubeculosus	1.06035103	1.17058932	0.30406271
Can f 1	Apis mellifera	0.99506445	1.1666758	1.26770931
Cul o 3	Culicoides nubeculosus	1.05540628	1.15963119	0.10902477
Leu vu	Glycine max	0.8107	1.1583678	0.97578135
Mus xp	Culicoides	0.91163213	1.15662565	0.71625959
Cul ob 8	Amaranthus cruentus	2.14149643	1.1560411	3.11816606
Ric c [Pollen]	Aspergillus fumigatus	1.11150929	1.15145499	0.66043195
Que i	Culicoides obsoletus	1.06380088	1.14768152	0.68299314
Der p	Setosphaeria rostrata	1.18086895	1.14404889	0.90864615
Gly m TI	Malassezia pachydermatis	1.10049049	1.13923311	0.74281825
C0167	Corylus americana	1.91331748	1.13666573	2.65059721
Mus m 1	Helix aspersa	1.10588127	1.13245551	0.68517108

Cul ob 8	Musa x paradisiaca	2.09019208	1.13099895	3.04465457
Cas s [Pollen]	Culicoides obsoletus	1.01789941	1.12823039	0.52480493
Hev b 1	Culicoides obsoletus	0.855107	1.11894712	0.47775036
Hev b 9	Dermatophagoides pteronyssinus	1.13929559	1.11486228	0.93689296
Alt a 1	Culicoides nubeculosus	0.90030068	1.11150837	1.11559379
Cul o 6	Triticum aestivum	0.8626512	1.10727665	0.42605585
Aca s	Bos domesticus	1.09673163	1.10700666	0.92028334
Asp v	Glycine max	0.99703376	1.10658837	0.59510167
Act c 11	Manioc	0.99941035	1.10599122	0.38763447
Ave s [Seed]	Culicoides nubeculosus	1.20553046	1.10338169	1.09327634
Cer si [Seed]	Alternaria alternata	1.09489793	1.10292411	0.74203812
Tri a [Pollen]	Culicoides obsoletus	0.81854499	1.09439618	0.72836986
Man e (peak)	Culicoides obsoletus	1.22409161	1.07844646	1.19945611
Hay d	Chaetomium globosum	1.03429989	1.07378375	0.56002452
Cul nu 2	Apium graveolens	0.98836347	1.06694174	1.07402212
Gly m Agglutinin	Taraxacum officinale	0.97097954	1.06636194	0.38518784
Cul o 3	Culicoides nubeculosus	0.99788094	1.06460102	0.37710376
Dre h	Ambrosia artemisiifolia	1.00802505	1.0601577	0.50858928
Equ c [Milk]	Culicoides obsoletus	0.86519532	1.04721139	0.53621681
Car b	Rumex acetosella	0.80710119	1.04128647	0.72650107
cul o 5	Ricinus communis	1.54465051	1.02339126	2.10730823
Bra n	Aeromicrobium ponti	0.62373436	1.02071954	0.52383366
Tar o [Pollen]	Artemisia vulgaris	0.99757647	1.0169596	0.65837621
Arm r HRP	Olea europaea	0.9008036	1.01677675	0.35132544
Mer hu 1	Juniperus virginiana	0.97335415	1.01274147	0.53041062
Ace s	Glycyphagus domesticus	0.65836176	1.00711105	0.93464981
Der p 10	Corylus avellana	0.88287857	1.00180011	0.22997879
Hev b 8	Dactylis glomerata	0.94574794	0.99326816	0.44588024

Cul ob 8	Castanea sativa	1.88748834	0.98826696	2.74425119
Ara h 1	Gallus domesticus	0.77358141	0.98576085	0.66480597
Bos d 5.0102	Culicoides nubeculosus	0.87596577	0.98395717	0.92295092
Hor v [Seed]	Dermatophagoides farinae	0.89974476	0.97875143	0.1822363
Cte f	Arachis hypogaea	1.03340761	0.97726192	0.8586864
Pen d	Helianthus annuus	0.91044738	0.97693371	0.43141795
Bla g 4	Culex pipiens	0.86695027	0.97616504	1.01846366
Cup a [Pollen]	Culicoides obsoletus	0.97995113	0.97138257	0.83418523
Culicidae Cul E	Merluccius capensis	1.58247039	0.97015899	2.19282677
Ory s [Seed]	Culicoides obsoletus	0.85426066	0.96775045	0.23372698
Bos d 11	Culicoides nubeculosus	0.74271245	0.9661221	0.68029461
Bos d 4	Culicoides obsoletus	1.15057049	0.96518417	1.52155116
Gly m 4	Microbacterium marinilacus	0.94569594	0.96451516	0.60152379
rAsp f 6	Culicoides nubeculosus	0.83822396	0.96371613	0.3491616
Cul o 7	Hordeum vulgare	1.76460453	0.96079863	2.54332399
Cla h	Lepidoglyphus destructor	1.03016871	0.95552389	0.9023704
Pla l	Actinidia deliciosa	1.0453148	0.95464887	0.96907055
Act c Chitinase_IV	Culicoides nubeculosus	0.91841376	0.95241319	0.60312316
Tri pr	Helix aspersa	0.96270476	0.95228802	1.02993242
Cul p	Culicoides obsoletus	0.52482428	0.94904132	0.49710076
Rum a	Drechslera spicifera	0.82484505	0.94860097	1.12996657
Tri a [Seed]	Culicoides obsoletus	0.84043166	0.94527555	1.20154516
Pru p 3	Ambrosia artemisiifolia	0.99248018	0.94374919	0.84034116
Ara h 1-NT	Fraxinus excelsior	0.78861302	0.94016087	0.27882692
Bos d 6	Artemisia vulgaris	0.97738819	0.9401359	0.83387105
Tri a [Seed]	culicidae	1.23406385	0.93689303	1.76410906
Pen i 1	Oryza sativa	0.98781559	0.93683056	1.01337658
†Cul nu 9	Actinidia deliciosa	0.90580017	0.93263403	0.5445339

Cul o 3	Culicoides nubeculosus	0.8698773	0.92559627	0.38283529
Art v	Mucor circinelloides	0.670976	0.92422485	0.47227741
Cul o 7	Rattus norvegicus	1.75325009	0.92165693	2.53602723
Ber e 1	Saccharopolyspora rectivirgula	0.91756562	0.92098087	0.65974974
Soybean AL3-native 1mg/ml	Culicoides obsoletus	0.96786051	0.91423491	0.82820227
Par j 2	Actinidia chinensis	0.9755782	0.91175699	0.85349949
Act d 1	Culicoides obsoletus	0.87008876	0.91106828	0.42547331
Api m [Venom]	Culicoides nubeculosus	0.82735066	0.91025521	0.28023932
Rhi n	Lolium perenne	0.79684164	0.90405677	0.51956936
Cul o 6 F10	Culicoides obsoletus	0.81312234	0.9036329	0.24098607
Gal d 1	Parietaria judaica	0.89130371	0.90218007	0.6452136
Mer mr 1	Glycine max	0.84340463	0.89714362	0.39189302
Cul n 2	Culicoides nubeculosus	0.57942732	0.88835251	0.42006928
Cul nu 2	Glycine max	0.83203859	0.88065252	0.40948462
Cul o 7	Secale cereale	1.71111536	0.87920596	2.48264908
Par o [Pollen]	Olea europaea	0.58584395	0.87835321	0.64793799
Bet v [Pollen]	Ananas comosus	0.75883936	0.87820086	0.21193926
Co180	tripholium pratense	1.71327915	0.87760512	2.48446376
Co15	Actinidia chinensis	1.36633589	0.87717871	1.82013267
Hev b 7.02	Triticum aestivum	0.71543376	0.86010092	0.79161795
cul o 2	Cupressus arizonica	0.82182931	0.85822727	0.84498406
Pru p 7	Malus domestica	0.71318755	0.85806929	0.26985941
Bla g 1	Aedes communis	0.70602904	0.85772018	0.3170376
Culicidae Cul A	Bipolaris sorokiniana	0.89051405	0.85573562	1.00483868
Cul nu 2	Salsola kali	0.69010038	0.85565264	0.58584476
Cul nu 1	Bos domesticus	0.90224006	0.85110313	0.98845671
IgE	Prunus persica	0.91017536	0.84928181	0.89531454
rAsp f 4	Culicoides nubeculosus	0.90732733	0.84066165	0.80021101

cul o 5	Culicoides nubeculosus	0.93600025	0.83685442	0.95342479
Mer ca 1	Avena sativa	0.800351	0.83456674	0.44480161
Cul nu 4 (GGC)	Bos domesticus	0.75595032	0.83086844	0.07710358
Asp r 1	Lupinus albus	0.79115605	0.83073054	0.4717526
Eur m 2	Alnus glutinosa	1.00533971	0.83068832	1.25719755
Ara h 2	Dermatophagoides pteronyssinus	0.75616077	0.82646711	1.11365957
Alt a	Bertholletia excelsa	0.9442596	0.82571461	0.97010317
Bla g 5	Alnus glutinosa	0.75250608	0.82244271	0.10055086
Act d 10	Parietaria judaica	0.60444417	0.82144088	0.38363138
Bos d 4	Culicoides nubeculosus	0.75964881	0.81785731	0.2359604
Cas s [Seed]	Culicoides nubeculosus	0.7701123	0.81359991	0.33806539
Amb p	Solanum lycopersicum	0.71014487	0.81228896	0.164137
Hor v [Pollen]	Culicoides obsoletus	0.54996059	0.81166351	0.37166446
Aur p	Parietaria judaica	0.76107096	0.80986527	0.50741158
Cul o 3	Glycine max	1.53033142	0.80944963	2.22242976
Cor a 9	Culicoides nubeculosus	0.72880252	0.80198254	0.21240742
Cul s	Controls	0.77115168	0.80102492	0.40421094
Aci g	Lolium perenne	0.64545197	0.79984114	0.27785874
Tri td	Culicoides nubeculosus	0.80676122	0.79971107	0.57079024
Cul nu 10.01	Merluccius merluccius	0.72005237	0.79766526	0.96474801
Culicidae Cul D	Actinidia chinensis	1.31560831	0.79556142	1.81526127
Aspe ni	Ambrosia psilostachya	0.84700624	0.79446922	0.77460132
Rat n 1	Hevea brasiliensis	0.92499035	0.79194922	1.35898816
Equ c Myoglobin	Bos domesticus	0.87341803	0.79157042	0.90957428
Ani s 3	Bromus inermis	0.72402673	0.79117361	0.79688634
Cha g	Aspergillus versicolor	0.77652322	0.78334565	0.50520064
Cul o 5	Culicoides obsoletus	0.78150052	0.78115116	0.53550029
Cul o 3	Alnus glutinosa	1.31656623	0.77940603	1.8977786

Alt a 1	Cladosporium herbarum	0.94060332	0.77298864	1.02265976
Zea m [Seed]	culicidae	0.8474436	0.77153288	0.8569107
Dau c	Hay dust	0.63055816	0.76782821	0.26000928
Cul o 3	Felis domesticus	1.2953504	0.76776322	1.87103934
†Cul nu 9	Actinidia chinensis	0.74990864	0.76588547	0.50463777
Cul n 4	Ovis aries	1.21433792	0.76492887	1.64584313
Cul ob	Merluccius hubbsi	1.49071324	0.76182251	2.17268381
Cul ob 8	Blomia tropicalis	0.62548285	0.76030072	0.34359633
Cul n 5	Gallus domesticus	1.29191528	0.75937153	1.84939744
Bla g	Rumex crispus	0.5364332	0.75382001	0.31370889
Dac g [Pollen]	Anisakis simplex	0.71627893	0.74459444	0.37826262
Gly d 2	Triticum aestivum	0.91509698	0.74071802	1.0154571
Set r	Aspergillus fumigatus	0.80875374	0.73571604	0.75795083
Act d 11	Olea europaea	0.66975359	0.73289281	0.06496034
Fel d 2	Betula verrucosa	0.73652408	0.730728	0.81362733
Cul o 7	Aspergillus nidulans	1.39621128	0.72975729	2.01715225
Asp f	Platanus acerifolia	0.84838482	0.72804773	0.88259628
Erw r	Vespa crabro	0.83999304	0.72678782	0.85065694
Cul n 4	Agrostis gigantea	1.22053202	0.72544464	1.69996716
Cul n 1	Hevea brasiliensis	1.38492443	0.7237537	2.00815298
Cul nu 10.02	Erwinia rhapontici	1.30162611	0.72295567	1.8797881
Der f 2	Dermatophagoides farinae	0.67057891	0.72244462	0.49505193
Act d 5	Plantago lanceolata	0.79969289	0.72015026	1.13706814
Lep d	Periplaneta americana	0.88773149	0.71973918	1.24442521
Ovi a [wool]	Hevea brasiliensis	0.55052266	0.71828665	0.26552436
Phl p 6.0101	Platanus acerifolia	0.52000811	0.7161966	0.39010929
Cul nu 4	Phleum pratense	0.8458582	0.71201729	1.22736497
Ama cr	Culicoides	0.68024983	0.71152464	0.97540779
Der f	Zea mays	0.57091071	0.71030047	0.44500087

Hev b 10	Cupressus arizonica	1.11617281	0.70650629	1.62160069
Cul o 6F10	Culicoides obsoletus	0.45337757	0.70470911	0.54386767
Cul n (new)	Dermatophagoides pteronyssinus	1.24525663	0.70342834	1.75485215
Co 13	Geotrichum candidum	1.23170255	0.70150251	1.77649114
Cul ob 3	Glycine max	1.20358115	0.69855596	1.74790434
Cul nu 1	Ulmus americana	0.64544651	0.69831018	0.38656321
Phl p 12.0101	Eucalyptus globulus	0.29747068	0.69619732	0.4547422
Cul n 3	Dermatophagoides pteronyssinus	1.30922731	0.69563523	1.90158947
Cul nu 10.02	Periplaneta americana	1.35648572	0.69483943	1.97976131
Cul n 3	Anthoxanthum odoratum	1.38418653	0.69258219	2.01975561
Culicidae Cul C	Prunus persica	1.23053915	0.68010257	1.79700283
Aln g	Hevea brasiliensis	0.68152184	0.67911281	0.54093314
Soya 11S (Claire) 10mg/ml	Black fly	0.69546095	0.67910981	0.75083959
Cul nu 10.01	Malus domestica	1.27116378	0.67510998	1.85426059
Eur m	Canis familiaris	0.63266413	0.67356727	0.87316195
Asp n	Parietaria judaica	0.70569634	0.67243564	0.88408166
Pla a 1	Culicoides obsoletus	0.34585355	0.67166564	0.3955863
Tyr p	Arachis hypogaea	0.75665344	0.67155901	0.79238106
Cul ob 6.02	Controls	1.23305476	0.67097585	1.76158354
Cul ob 6-like	Acinetobacter gerneri	1.18983313	0.66916639	1.67497502
Cul n 4	Aspergilus niger	1.14514168	0.66795175	1.67310503
Cul o 5	Penicillium expansum	0.74731012	0.66405899	0.72680298
Cul o 3	Cynodon dactylon	1.16269611	0.66388769	1.69822773
Der p 1	Bos domesticus	0.70886869	0.65733016	0.99731073
Pho d 2	Culicoides obsoletus	0.4402912	0.65599224	0.35793771
Bos d 5	Alternaria alternata	0.49991034	0.65305759	0.26853431
cul o 2	Epicoccum nigrum	0.51233857	0.65139495	0.74066314
Gal d 3	Bos domesticus	0.58133885	0.65048851	0.21515697

Aed c	Culicoides nubeculosus	0.78733466	0.6489361	0.88845831
Der f	culicidae	0.47090362	0.64614239	0.6724546
Bos d [Epithelium]	Hevea brasiliensis	0.82587254	0.64596203	1.20847562
Aln g	Actinidia deliciosa	0.43055989	0.64570782	0.29139555
Ani s	Culicoides obsoletus	0.69647045	0.64426133	0.66253378
Bos d 12	culicidae	0.52776911	0.64301064	0.78379436
Cul n	Salix lasiolepis	1.12528551	0.64217307	1.58135243
Car p 1	Phoenix dactylifera	0.60745989	0.63413375	0.33109572
Ant o [Pollen]	Triticum polonicum	0.53752177	0.62950562	0.54365383
Lin us	Culicoides obsoletus	0.74733257	0.61562122	0.8660376
Cul n 1	Dermatophagoides farinae	0.71493678	0.61128641	1.03997854
Gal d [Egg Yolk]	Culicoides obsoletus	0.62408617	0.61114932	0.64371959
Mala p	Aspergillus restrictus	0.36026337	0.6100451	0.53768539
Pla a [pollen]	Carica papaya	0.50215106	0.60888743	0.67610863
Vesp c	Culicoides nubeculosus	0.59941239	0.60806437	0.37595979
Urt d [Pollen]	Controls	0.78045172	0.60697185	0.97703401
Ovi a [Epithelium]	Blattella germanica	0.78176183	0.60631729	1.03767784
Fus s	Anisakis simplex	0.63576258	0.59432674	0.63679403
Can f 3	Culicoides obsoletus	0.42593287	0.59091798	0.31814801
Jun v	Phleum pratense	0.56857502	0.58846564	0.31004525
Rhod k	Canis familiaris	0.79945735	0.5882156	0.97518751
Rum cr	Apis mellifera	0.4481874	0.58290561	0.22955016
Zea m [Pollen]	Culicoides obsoletus	0.43077642	0.57691527	0.43787619
Phl p	Canis familiaris	0.35556257	0.57618092	0.39289053
Cul o 6	Equus caballus	0.81199353	0.57231122	1.02541003
Sal la	Alternaria alternata	0.55371043	0.57178484	0.44375305
Cor a PG	Dermatophagoides pteronyssinus	0.54625027	0.57122445	0.27248538
IgE	Cynodon dactylon	0.75503085	0.56969465	1.09029093

Cul n 11	Eurotium amstelodami	1.05076867	0.56810572	1.53237566
Equ c 3	Aspergillus fumigatus	0.51840389	0.56598789	0.09029427
Fes p [Pollen]	Dermatophagoides farinae	0.4895184	0.564703	0.34039094
Man e (flow through)	Bos domesticus	0.72336845	0.56422258	0.83367896
Bos d 9	Culicoides	0.86875	0.56333205	1.15645704
Pla a 2	Culicoides nubeculosus	0.56641707	0.5583329	0.41056697
Ara h 1-CT	Gallus domesticus	0.46899341	0.55463477	0.16089271
Cul nu 6-like	Hevea brasiliensis	0.95854792	0.5545038	1.36069712
Act c 5	Culicoides obsoletus	0.39444823	0.54845793	0.22367483
Cul o2P	Eupatorium capillifolium	0.91879031	0.54780212	1.28031462
Cul nu 3	Secale cereale	0.99298291	0.5375138	1.44409848
Bos d 5	Culicoides nubeculosus	0.49052411	0.53661504	0.0805725
Cyn d [Pollen]	Dermatophagoides pteronyssinus	0.40229396	0.53529019	0.20420723
Che a	Mus musculus	0.52187411	0.53503511	0.32743775
Amb a 1	Euroglyphus maynei	0.46108823	0.53463717	0.1844543
Gly m	Culicoides nubeculosus	0.53624487	0.53139311	0.70918498
Fag g	Anisakis pegreffii	0.54622743	0.53047148	0.47486654
Der p 23	Lepidoptera	0.63136274	0.52784527	0.81633624
Cul nu 6-like	Betula verrucosa	0.95801081	0.52779791	1.3987924
Culicidae Cul B	Fagus grandifolia	0.94980573	0.52597857	1.34463495
Cul nu 10.01	Culicoides nubeculosus	0.76728818	0.52586617	1.11730984
Pen ch	Arachis hypogaea	0.49357376	0.52020775	0.69855817
Cul nu 4	Phleum pratense	0.95726635	0.51072538	1.37120986
Api g [Root]	Culicoides obsoletus	0.51669482	0.510046	0.57676093
Cul n 2	Holcus lanatus	0.19394164	0.50918604	0.30365981
Amb a	Bos domesticus	0.55114523	0.50824312	0.49542463
Cul o 2	Culicoides obsoletus	0.72771207	0.50590305	1.049575
Mus m [Epithelium]	Aspergillus fumigatus	0.44170139	0.50431413	0.37527674

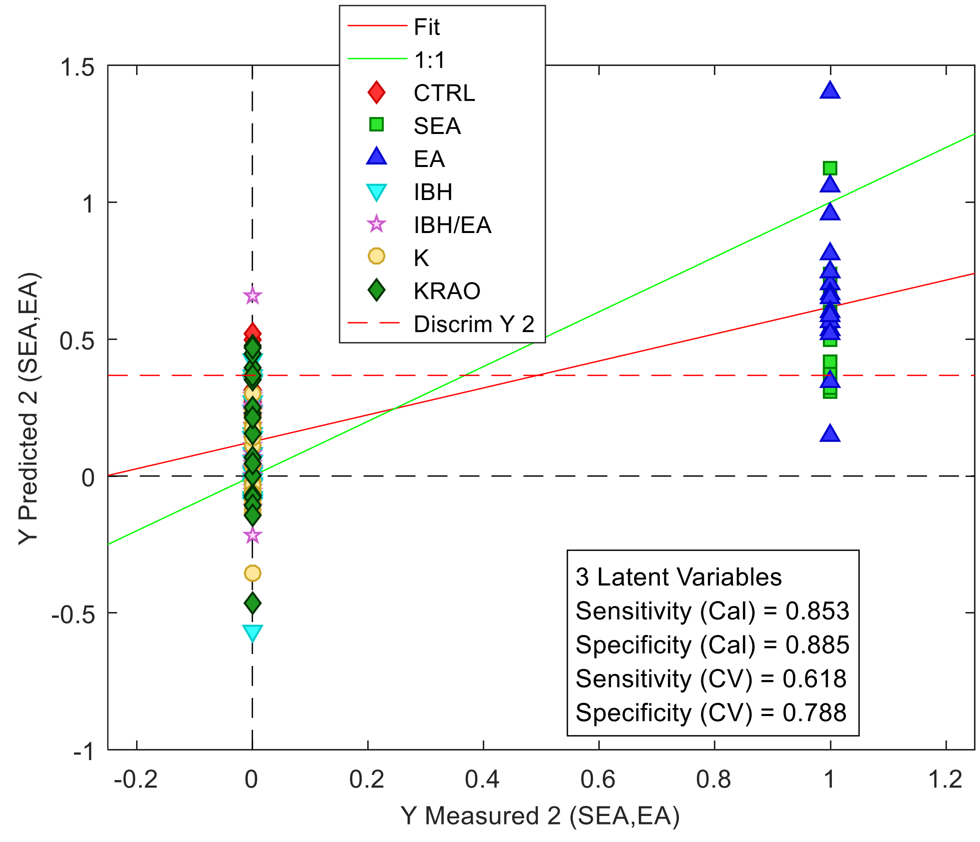
Hel a [Seed]	culicidae	0.37218646	0.49167315	0.39568292
Zea m [Seed](BIAL)	Culicoides obsoletus	0.33708854	0.48588005	0.42397055
Api m 1	Blattella germanica	0.43345659	0.48438964	0.11240803
Art v 4	Castanea sativa	0.60491794	0.48390866	0.75913717
Gal d 2	Culicoides obsoletus	0.46609465	0.48123036	0.58107222
Cul o 8 C11	Culicoides nubeculosus	0.42369718	0.47909191	0.45310939
rAsp f 3	Actinidia deliciosa	0.44575386	0.4786181	0.19573555
Ani pe	Culicoides obsoletus	0.61335226	0.478587	0.714952
Bos d [Milk]	hay dust	0.43128315	0.47731664	0.27342986
Sola t 1	Linum usitatissimum	0.34763311	0.47445852	0.39466
Cul ob 6-like	Controls	0.87424871	0.47061512	1.27643582
Cul o 2	Gallus domesticus	0.83362625	0.46513398	1.17729456
Cul o 4	Helianthus annuus	0.31392806	0.4641644	0.46570357
Cul nu 10.03	Culicoides nubeculosus	0.8686915	0.46241211	1.25789793
Pru p Hevb5-like	Alternaria alternata	0.40546523	0.45973693	0.45585003
Gly d	Drechslera halodes	0.55888531	0.45848129	0.77541644
Phl p 2.0101	Rattus norvegicus	0.53700073	0.44991143	0.69329352
Mal d 1.0108	Culicoides nubeculosus	0.3131152	0.44734568	0.2424943
Ana c 2	Blattella germanica	0.26720119	0.44631443	0.29696349
Co 60	medicago sativa	0.67718524	0.44294764	0.9897024
Moth Heterocera 10mg/ml	Anisakis simplex	0.44349185	0.44054729	0.33239679
Str a	Blatta orientalis	0.60692482	0.43912619	0.74803097
Bos d 8	Culicoides obsoletus	0.70683218	0.42357551	0.98204361
Bip so	Rhizopus nigricans	0.59401986	0.42324959	0.86960861
Med s [Pollen]	Salsola kali	0.34718727	0.41947642	0.20096121
Cul o 2	Quercus ilex	0.79726236	0.41837857	1.14776726
Equ c [Epithelium]	Blattella germanica	0.43148192	0.40735818	0.37278483
Cul ob 6.01	Phleum pratense	0.79163315	0.40602605	1.14787637

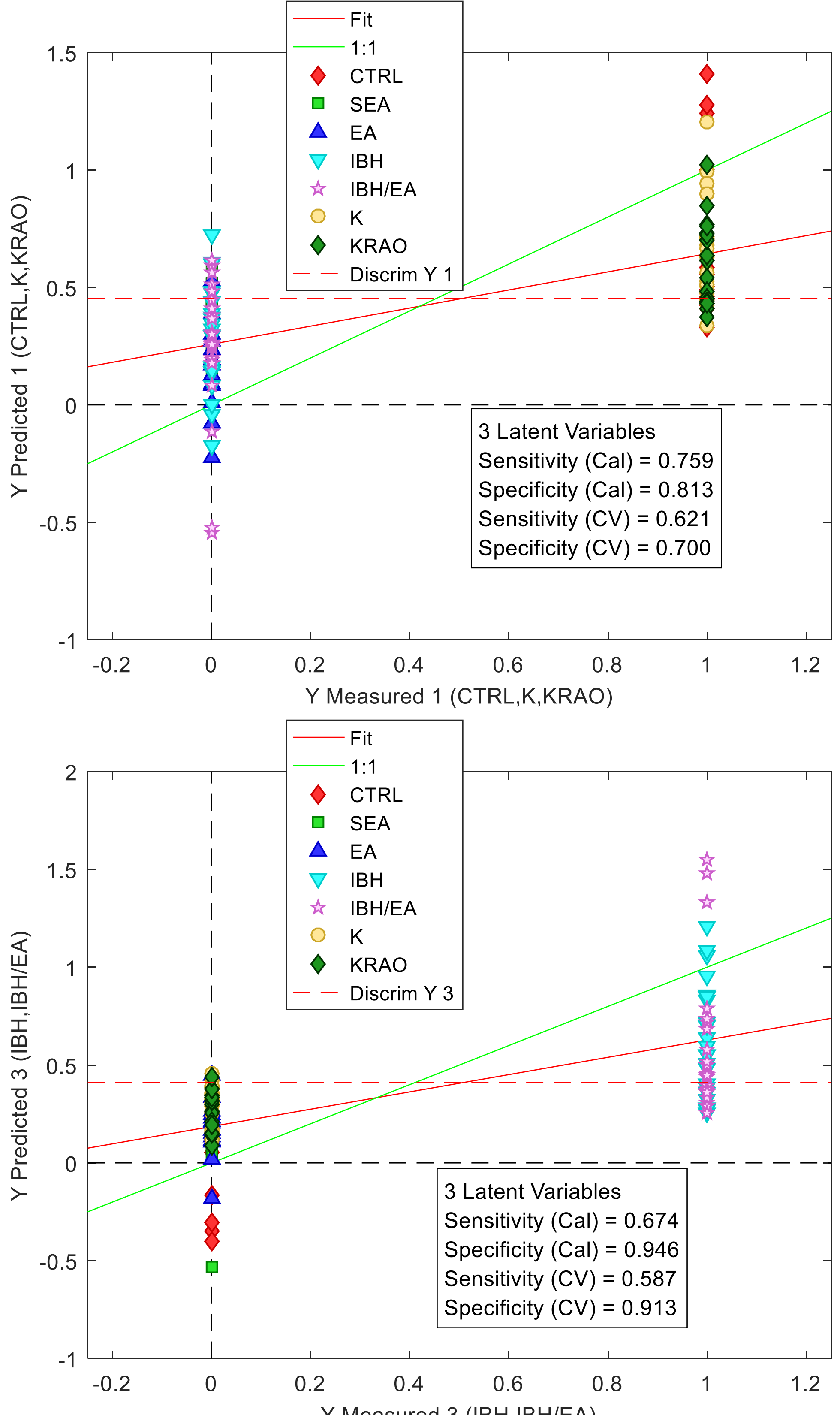
Dre s	Festuca pratensis	0.48165801	0.40244554	0.68746472
Hol l [Pollen]	Artemisia vulgaris	0.36281336	0.40129765	0.20304703
Ave s [Pollen]	Hevea brasiliensis	0.35442706	0.40040679	0.13653718
Cul ob 6.01	Kineosporia rhizophila	0.65911522	0.39661057	0.9545292
Mal d [Fruit]	Culicoides nubeculosus	0.60795447	0.3909027	0.84758627
Phl p 7.0101	Hordeum vulgare	0.49820104	0.38907824	0.65683188
Fra e	Cladosporium herbarum	0.31454551	0.38763129	0.13061994
Cul nu 10.03	Culicoides nubeculosus	0.66673234	0.37641154	0.94568382
Ani s 1	Brassica	0.51412546	0.37441675	0.74477982
Cul n 8	Aureobasidium pullulans	0.35201731	0.36559893	0.45959765
Cul o 8 (15KDa)	Penicillium digitatum	0.69831184	0.36318837	1.0084153
Cul nu 4	Chenopodium album	0.29126395	0.35468653	0.19539255
Sal k 1	Culicoides obsoletus	0.38961891	0.35059882	0.52920655
Sec c [Seed]	Culicoides obsoletus	0.37470581	0.34608173	0.38660745
Per a	Culicoides obsoletus	0.24334242	0.34294288	0.21500088
Cor a [Pollen]	Manioc	0.32857533	0.3421173	0.18948681
Cor a 1.0103	Hevea brasiliensis	0.26657603	0.34051458	0.14601457
Cy3	Culicoides obsoletus	0.4637856	0.33324342	0.63693853
Cul o 2	Fusarium solani	0.66133696	0.33078573	0.96495088
Der p 7	Arachis hypogaea	0.53365109	0.32962991	0.74350813
Bro I [Pollen]	Prunus persica	0.28991654	0.32515651	0.23040831
Lol p 1	Daucus carota	0.53532099	0.32113149	0.76111807
Culicidae Cul F	Prunus persica	0.29012814	0.31833564	0.03967664
Sor h	Betula verrucosa	0.44767903	0.31620205	0.63390473
Bos d CA	Penaeus monodon	0.23539108	0.31554277	0.32753716
Co147	Bos domesticus	0.57312059	0.31334378	0.81734115
Alt a 6.0101	Hevea brasiliensis	0.21224822	0.3082565	0.31778374
Api m 4	Aspergillus fumigatus	0.3555207	0.30625008	0.39997832

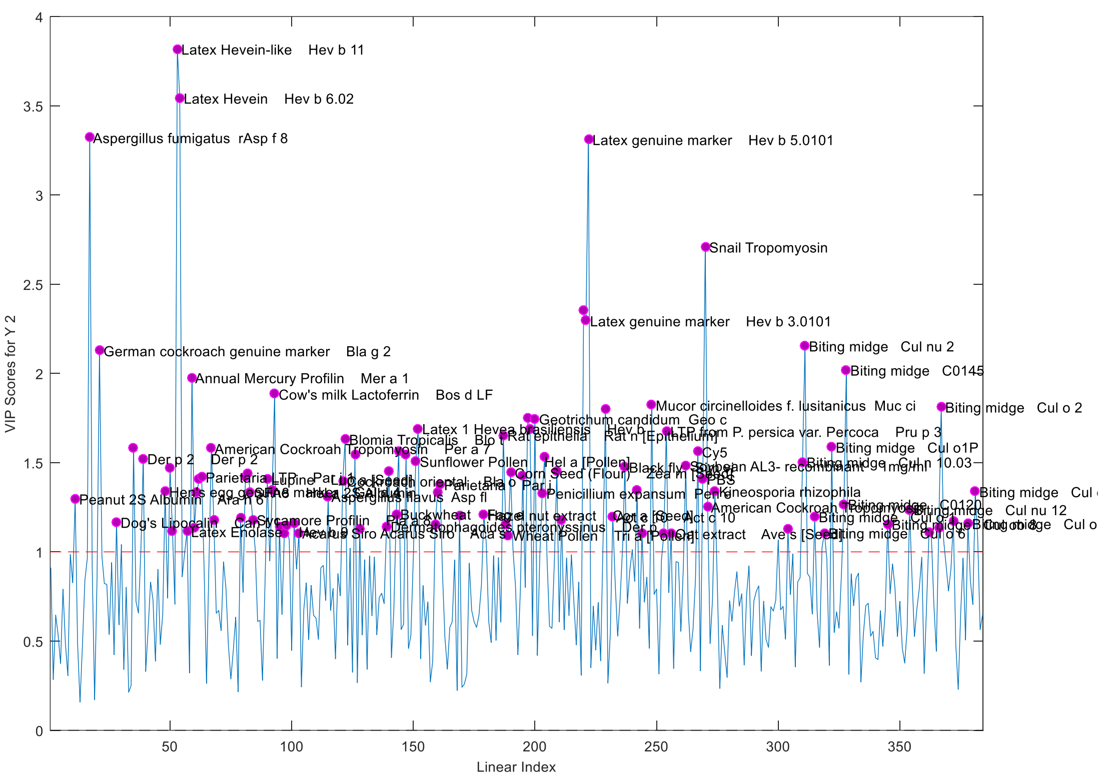
Mic m	Tyrophagus putrescentiae	0.57051507	0.29610876	0.83179635
Tri a Gliadin	Corylus avellana	0.34113516	0.28751801	0.43947564
Aln g	Urtica dioica	0.39348362	0.28421478	0.57332485
Tri tp	Culicoides nubeculosus	0.2993503	0.27983574	0.29366198
Lol p [Pollen]	Equus caballus	0.30482767	0.27078189	0.4451167
Cul nu 2	Avena sativa	0.26311929	0.27044667	0.30639784
Cand a	Culicoides obsoletus	0.34898414	0.26633687	0.41080864
Sac r	Equus caballus	0.22210117	0.26454395	0.27020139
Par j 3	Glycyphagus domesticus	0.49722661	0.26290905	0.71714006
Sec c [Pollen]	Arachis hypogaea	0.27953171	0.25917759	0.31928662
Cyn d 12	Triticum aestivum	0.26047584	0.25165212	0.36497811
Agr g [Pollen]	Gallus domesticus	0.23203486	0.24281748	0.33964379
Sal k	Corylus avellana	0.18618728	0.24115618	0.11849771
Aer p	Penicillium chrysogenum	0.1902219	0.23482113	0.09393167
Bos d LF	Culicoides	0.23965199	0.22908846	0.19264291
Poa p	Hevea brasiliensis	0.21105562	0.22271094	0.25731522
Pop a	Penaeus indicus	0.31760692	0.21539556	0.43915707
Cup a 1	Sorghum halepense	0.18155692	0.21308705	0.06956999
Bet v 1.0101	Streptomyces albus	0.14741884	0.1713855	0.19232114
rAsp f 1	Thermoactinomyces vulgaris	0.11875442	0.15805182	0.16496727

Appendix F: PLS-DA combined modelling

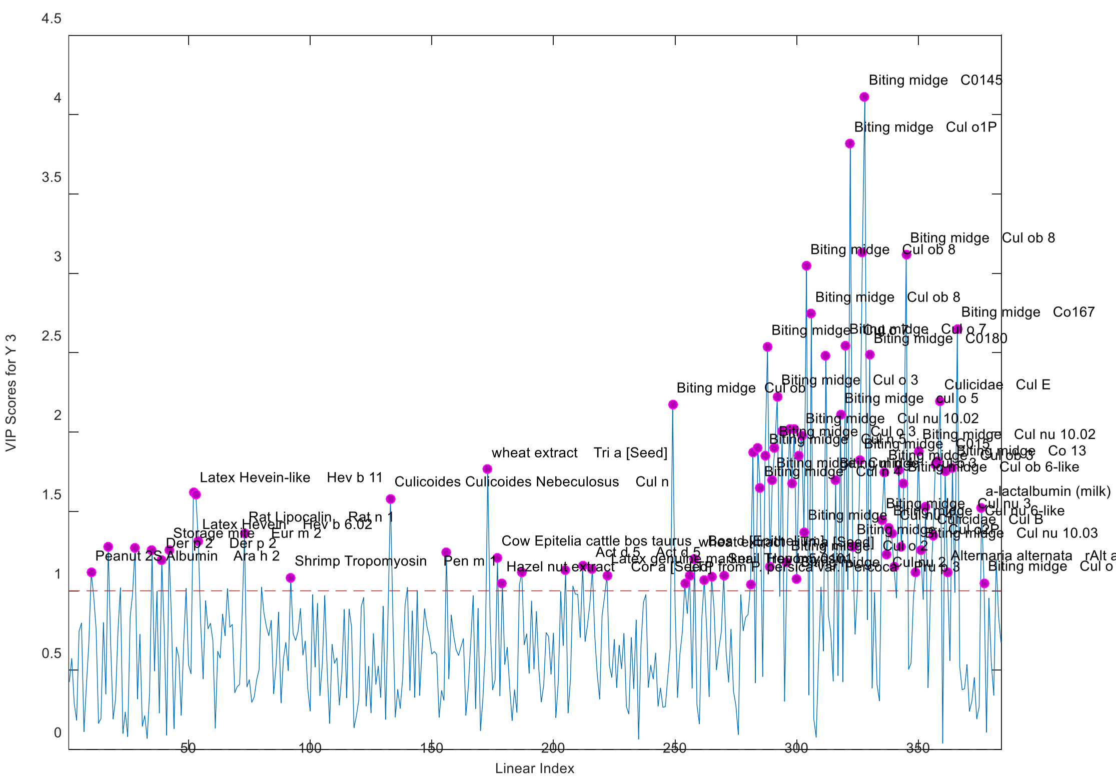
Using the amalgamated file and excluding urticaria and unknown controls:
For SEA/EA




Fit
1:1
CTRL
SEA
EA
IBH
IBH/EA
K
KRAO
Discrim Y 1
3 Latent Variables
Sensitivity (Cal) = 0.759
Specificity (Cal) = 0.813
Sensitivity (CV) = 0.621
Specificity (CV) = 0.700
Y Predicted 1 (CTRL,K,KRAO)
Y Measured 1 (CTRL,K,KRAO)
Fit
1:1
CTRL
SEA
EA
IBH
IBH/EA
K
KRAO
Discrim Y 3
3 Latent Variables
Sensitivity (Cal) = 0.674
Specificity (Cal) = 0.946
Sensitivity (CV) = 0.587
Specificity (CV) = 0.913
Y Predicted 3 (IBH,IBH/EA)
Y Measured 3 (IBH,IBH/EA)

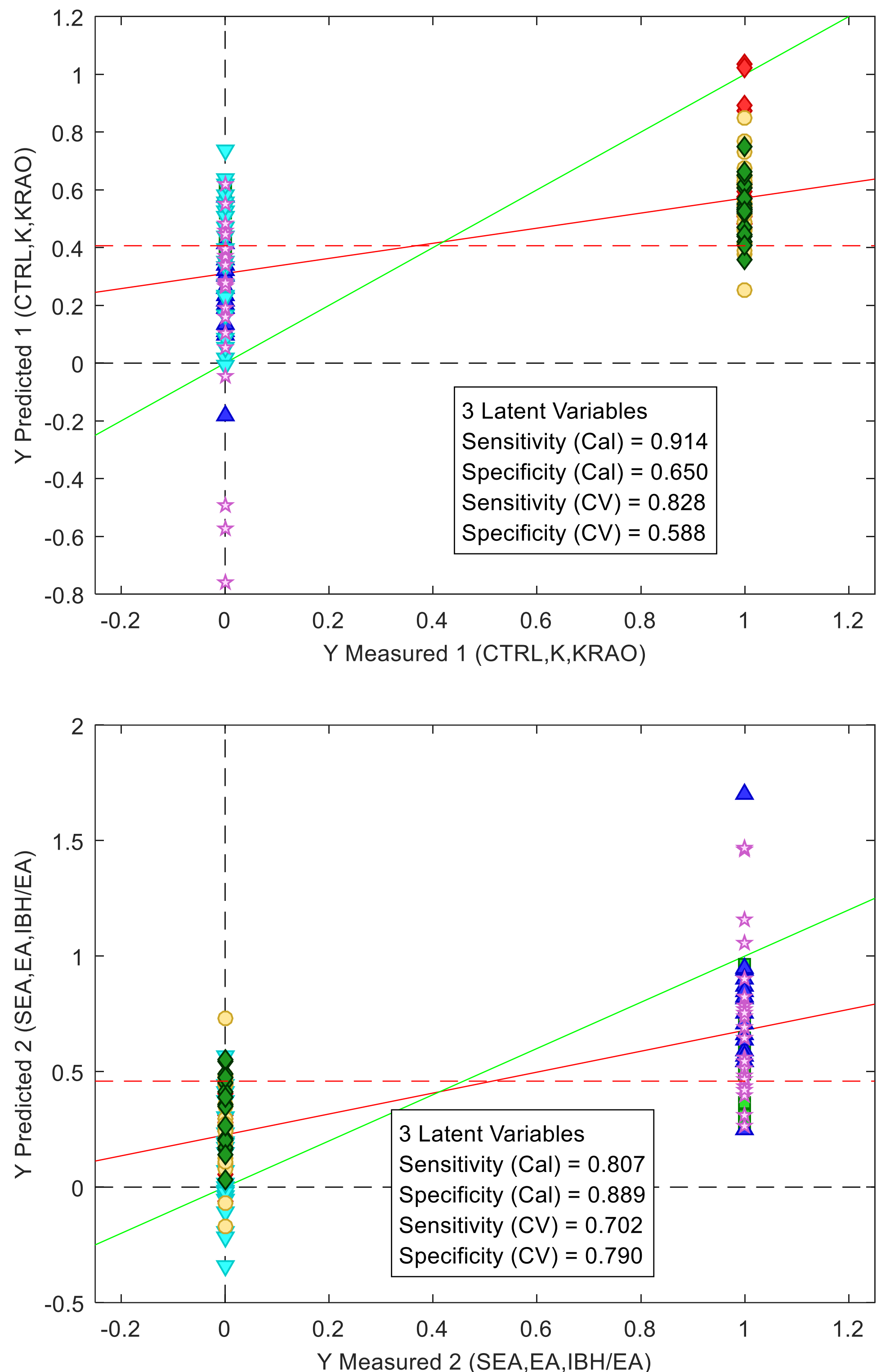


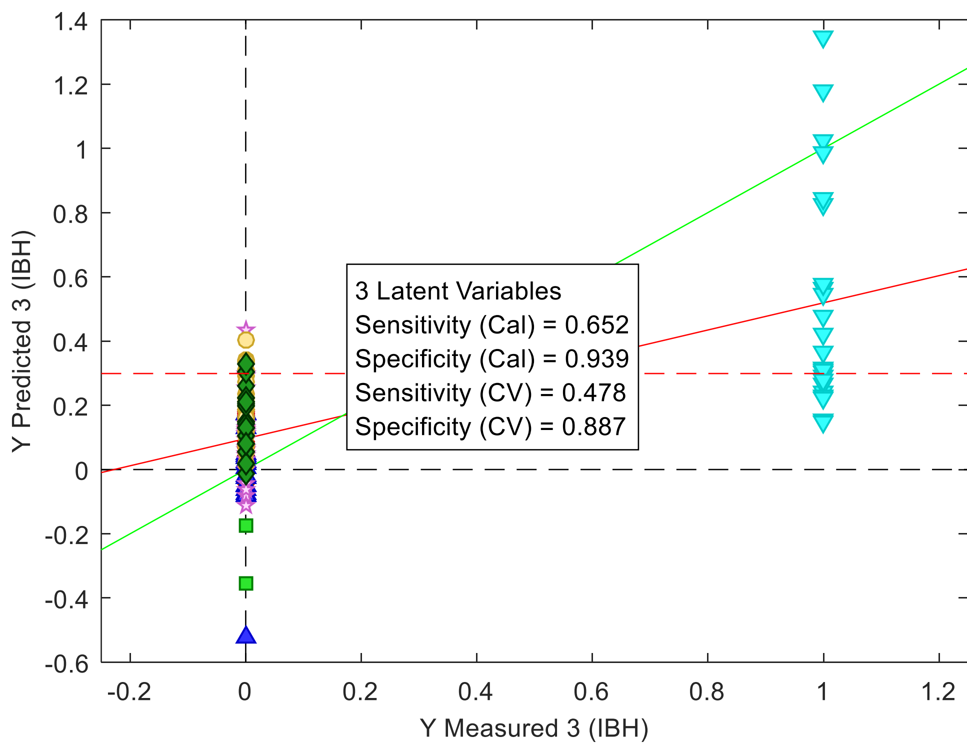
For IBH



Decluttered

Using only sEA VIPs for classification:




3 Latent Variables
Sensitivity (Cal) = 0.652
Specificity (Cal) = 0.939
Sensitivity (CV) = 0.478
Specificity (CV) = 0.887
Y Predicted 3 (IBH)
Y Measured 3 (IBH)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.2
0
0.2
0.4
0.6
0.8
1
1.2

Appendix G: hay treatment raw data

SERUM AC	DRY AV	C SOAKED	AC STEAMED	F SERUM A	F DRY AV	F SOAKED A	
Sycamore G	7700	1863	1444.5	2437.5	59.5	361	643.5
Apricot Pr	523	-20.5	-104.5	3	3300.5	70	127.5
Hazelnut Eu	355.5	758.5	583	774.5	1214.5	439	525
Chestnut Po	534.5	153.5	19	92.5	10073.5	1809	4741.5
Rye Grass p	11726	414.5	127.5	714	4139.5	2877.5	3054.5
Barley Polle	13489	804	446.5	1505.5	122	47	17.5
Timothy ph	3634.5	437.5	74	267	502.5	678	499.5
Malassezia	2311	492	444.5	365.5	201	75	140
Juneky blue	5390.5	444	108.5	529.5	257.5	87.5	63.5
Orchard Da	3044	193	-10	268.5	232.5	357	365.5
Hazel pollen	5877.5	1492.5	1314	1433	58.5	131	225
Aureobasid	5262.5	556.5	619	529	37.5	105	18.5
smoked had	37	-260.5	127.5	332	3397	86	106.5
Corn Seed (	277.5	777.5	381	835.5	2035.5	510.5	895.5
Hamster Ep	590	470	171.5	352	11658	1568	2488
Bromelain C	17471	1084	759.5	1477.5	5005	223	167
Penicilium n	4047	931.5	658	692.5	172.5	40	66
Cy5 1mg/	2402.5	2216	2479.5	1429	46	178.5	191.5
Oak pollen	1794	119	7.5	-86.5	137.5	85	60
Palm Profili	983	1408	955	1148.5	5500	62	65
Rhizopus ni	1924	554.5	705.5	542.5	14891.5	2537	5796.5
Alternaria a	1191.5	323.5	105.5	84.5	-25	-10.5	25.5
American C	570	920.5	1099	1440.5	160	118.5	95
Tyrophagus	5602.5	5172	5651	3098.5	3494.5	10	78.5
Aspergilus n	9103.5	3341.5	2435	2475.5	5326.5	2200	2625.5
Artichoke	-208.5	-534.5	-613	-544.5	132	274.5	88.5
Cy5	256	204.5	85.5	132.5	10610.5	133.5	297
Johson gras	15070.5	1195	504	1721	446.5	192.5	140.5
Cow Epiteli	1178.5	926.5	595.5	1121.5	180	212.5	213.5
Fennel dog	7982	334	303.5	787.5	177.5	113.5	69.5
Chaetomiu	1778.5	1079	1381.5	1248.5	211.5	371	424
Cow's milk	65	201	40.5	83	3445.5	1465	1395.5
Kineosporia	889.5	561	509	657.5	596	208	139
Aspergillus	322	511.5	362.5	755	10885.5	5788	6320.5
pear extrac	53	-48	37	224	295.5	145.5	155.5
Margerite (	682.5	382.5	286	196	9176	321.5	421.5
Peanut extr	-34.5	1028.5	712.5	728	1113	137.5	202.5
Peafowl eg	178.5	663.5	356.5	284	114.5	52	-16.5
Chestnut Se	1431.5	155.5	35	191.5	140.5	108.5	83
Setosphaer	2705	1334	1305.5	1147	517	253.5	200.5
Hazel nut e	32.5	1181	585	437.5	58.5	27.5	103.5
Helminthos	3640.5	361	266.5	175	238	245.5	158
Celery stalk	335.5	125	15	248.5	388	209	260.5
Snail Hel a	2558.5	636	550.5	1130	57	253.5	401.5
broad bean	64.5	147.5	30.5	253.5	597.5	110.5	99.5

soy bean ex	1961	1143.5	1418.5	1406.5	96	177	190.5
Red top gra	5611	213	75.5	222	163.5	96.5	72
Barley Seed	1844.5	644.5	745.5	1339	900	149	110
Corn Pollen	7965.5	546	363	1169	313.5	100.5	104.5
Sunflower P	5209.5	535.5	145	809	638	359.5	294
Shrimp Pa	206	276	881.5	957	11953.5	1338	1494
Maple soft	1171.5	387.5	194.5	278.5	934	51.5	202.5
Latex 1 Hev	778	303	182.5	150	368.5	326	335
Arroyo Will	406	120	1	165	200.5	429.5	266
Fescue mea	5108.5	473.5	24	277.5	2802.5	359	519.5
Cow's milk	97	658.5	503.5	568	152.5	10	-54
Rye, Perenn	6803.5	451.5	45.5	487	319	83	112.5
Cypress gen	7787.5	834.5	470	1512	301.5	-12	41.5
Lamb quart	3804	509.5	184	480.5	68.5	112.5	53.5
Garlic All	851	179.5	109	266	198.5	7	6
Peanut 2S A	97.5	814.5	300.5	469	54	218	132
Common So	452	249	397	334	25	46	51
Tangerine,	2002	114.5	49.5	33	222.5	369.5	279
Par j 2 Pa	2364	187	101	101.5	574	250	192
rBer e 1 1	185	1596	1330.5	521.5	-3.5	42	-35
Wheat Poll	3641.5	-8.5	25.5	278.5	1620	105.5	147.5
Mustard S	-457	-1061.5	-910	-828	33.5	-31	167.5
Dog (?) IgA	-47	18	-34	98.5	1931	604	958.5
Cat albumin	38.5	51.5	40.5	137	504	819	590.5
Erwinia rha	1277.5	1034	1046	1172	2763	454	562
Peanut 7S V	419	1144.5	1041.5	831.5	2566.5	501	453
Aspergillus	1640.5	1320.5	1796.5	1869	181	221.5	416.5
Beech Ame	1134.5	224	99.5	106.5	122.5	59.5	15.5
Potato So	16142.5	1810	1519.5	2849.5	2017.5	382	511
Blue Musse	2016.5	169.5	312	196.5	1668.5	273.5	358.5
Latex Enola	1788.5	3071.5	1788	2283	90	129.5	85.5
Rice Ory s	355.5	-41	7.5	66.5	155.5	39	125
Potato So	10360.5	908.5	320.5	1281	693.5	149	155
Wild Strawb	-1140	-1662.5	-1386	-1473	-18	52.5	41
Peanut 7S V	1019.5	2262	1907.5	906.5	207	351.5	248
Euroglyphu	4255	7898	4407	6538.5	3299	2329.5	2159
Apple Ma	-291	-474.5	-547.5	-804	-9.5	83	7.5
Plantain en	783	97	77.5	215	1478	261.5	261
Oat extract	1025	151.5	202.5	135.5	4674	7521.5	6309
Rusian This	3722.5	884	946	1277.5	948	1753	1562.5
Sheep's mil	140	868	828	1022	640.5	1113.5	974
Velvetgrass	9251	385.5	242.5	648	4185	2881.5	1344.5
Tomato pul	2721.5	298.5	263.5	380.5	4	19	-22
pistachio ex	2247	1202	1366.5	1573.5	1358	650.5	915.5
Bee venom	11459.5	3108	2545	1451	130	182	39
Shrimp Tro	-50	938	554	867	158	70	-5.5
Oak English	1439.5	155.5	-53	-63	774.5	956.5	653
Empty	1052.5	22.5	62	254	147	528.5	222.5
Cedar Red J	-6.5	-167	-213	-146.5	126.5	183.5	172.5

Onion All	1724	358	308	272.5	801	135	51
Shrimp Tro	83.5	732.5	463	772	166	56.5	4
Acinetobac	1800	1152	610.5	553	384.5	238	337.5
ragweed, S	1363	227.5	111	392	92	-6.5	19
Penicillium	2245	1156	1404	1362	9134.5	84	247.5
Parietaria	1572.5	456	241	330.5	1056	1988.5	1571.5
Ragweed, W	440.5	55.5	-23	-14.5	478.5	612	669
Bee venom	1879	343	256.5	397	2856.5	1250	1612
Phl p 2 Ph	19	77.5	61.5	125	872.5	69	107
Human IgG	338.5	495.5	565.5	490	117	175	69.5
Anisakis (m	1791.5	3347.5	1918	2856.5	1152.5	1549	1189
Latex genui	756.5	2001.5	556.5	429.5	161.5	152	150
Cow's milk	202	665.5	488.5	1093	130.5	228	73.5
AgO+ AgO	490.5	864	450	295.5	253	16	57.5
Bermuda C	17823.5	4637	3157.5	7297.5	81.5	168	90
Grass Smoo	3089.5	94.5	70	60.5	1539	876.5	999.5
Dandelion T	474	93.5	7	-22	846.5	1442	1434
Rye Cultiva	3420.5	234	59.5	358.5	321.5	403	374
Drechslera	2826.5	1564.5	1574	1373	2250	803.5	1203.5
Cow's milk	324	659.5	352.5	476	2936.5	3925.5	2950.5
Eucalyptus	118	15	69.5	40.5	-1	18.5	35.5
Goat's milk	217.5	751	688	867.5	4325	1865.5	2205.5
Hazelnut Am	68.5	107	-62	0.5	368.5	262.5	117.5
SFA8 Hel	48	405	234.5	99.5	248.5	119	192.5
Olive pollen	874.5	99	10	96	2720.5	279.5	361.5
cu07 cu07	177	336.5	128	219	77.5	228	224
Hazelnut Am	610	478.5	374	262	529.5	768.5	534.5
Cladosporiu	2029	163	161	307	220	357.5	312.5
Aspergillus	3524.5	1318	1313	1230	465	213.5	129
Human IgM	1046	479.5	908	643	-15.5	-37	-56
coconut fle	64	162	90	102.5	2006.5	576.5	795.5
Sweet Vern	11692.5	687	245.5	1226.5	127	199.5	361.5
Olive Olea e	1094.5	209	134.5	136	189	245	166
Donkey's m	145.5	520	339.5	386.5	1107.5	1811.5	1509
fennel Gree	341.5	211.5	49.5	310	655.5	235	227
Candida C	14638	10220.5	8278	11166.5	33	1	5.5
Ara h 2 1m	-77	319	139	285	319	228	205.5
Pheasant e	268.5	178	176.5	41.5	1865.5	1418.5	1199.5
Monk fish (	322	450.5	59	112.5	193.5	286.5	250
Bee venom	175	47	4.5	1	38.5	-12	174
Pheasant e	339	484	368.5	583	449	376	159.5
Peanut 2S A	-78	614.5	688.5	165	71	131	67
Pellitory, w	772.5	-14.5	37	63.5	187	104.5	86.5
IgE one eigh	56.5	145.5	81.5	208.5	2882	1577.5	1576.5
Asparagus	452.5	259.5	59.5	279.5	320.5	490.5	239.5
Bean castor	720	150.5	107.5	77	74.5	27	28.5
Saccharopo	395	650.5	486.5	319	1126.5	645.5	982.5
Quail egg w	368.5	806	603.5	744.5	137	308	148
Streptomyc	2609.5	2168.5	2847.5	2838.5	349.5	75	340

Soya 11S (C	50.5	315.5	161	401	384	527	444
Mucor circi	1013	214	183.5	118.5	163.5	90.5	138.5
Mite Tropo	-48	65	292.5	476	248	310.5	419
Ananas (Pin	-489	-937.5	-812.5	-603	1454.5	241	235.5
Dock Yellow	18700.5	8919.5	6274	12224	272.5	-11	-19
salmon Sa	-15	1197	407.5	301	3.5	102	63.5
yeast extra	182.5	-16.5	26.5	18.5	458	94	-30.5
Tunafish T	-59.5	140	-4.5	60	2771.5	1436	1336
celery extra	-727	-1198	-904.5	-1018	1034	316.5	252.5
rye extract	299.5	205	171.5	355	196	294.5	158
Snail Tropo	661.5	1307.5	1120.5	1454.5	7192	260	377.5
Acarus Siro	96.5	546.5	266	104	90	153.5	79.5
Horse Mare	103.5	560	410	193	8.5	-32.5	-27.5
ELM Ameri	351	68	31	-94	928	354.5	213
CO23 CO2	740.5	1376	1505.5	1495	-32	5	-20.5
CO110 CO	55	100.5	41	119.5	4.5	27.5	-45.5
sesame Se	289	163.5	16.5	287	327.5	304.5	585.5
Moth Heter	1998	2504.5	1791.5	1898.5	122	227.5	248
Cockroach,	1898	681.5	426	820	269	161.5	191.5
Human IgA	2804	3806	3025.5	3632	383	278	214
Anisakis ge	67.5	-58	-172.5	-160.5	87.5	125.5	66.5
Dog's Album	40.5	175.5	90	167	121	163.5	152.5
Ragweed ge	45	553	165	220	153	82.5	40
Latex Heve	850	1067	607	917	284	400.5	475.5
pork Sus s	65.5	-19	52.5	-65	135	36	79.5
Cu06 Cu0	247	484	434.5	410	-25	41.5	-53
Tomato see	256	393	178.5	8	743	464.5	425
Helmeted G	384	126.5	1	-131	290.5	451	238.5
Water Buffa	138.5	645	706.5	961	47	63.5	71.5
Penicillium	2452.5	1243.5	1232	1065	54.5	51.5	60.5
Mugwort g	-32	72	-2	-103	-22	16	27
Cu04 Cu0	488	2738.5	883	694.5	148.5	79	81.5
Camel's mil	245	707.5	307	672	212.5	163	194.5
Parietaria L	842	263	94	450	94.5	163.5	138
lamb extrac	189.5	233.5	218.5	143	982	1170	703.5
Peanut 7S V	178	577.5	681	763.5	553.5	300.5	184.5
Lentil Len	125	622.5	565	144.5	134.5	246	159.5
Anisakis (O	2466	4336.5	3060.5	4139.5	135.5	89	77
turkey egg	250	46.5	72	-152	371	297.5	463
Flax Lin us	-51.5	50.5	25.5	66.5	403	233	152
Rice Ory s	1849	373.5	-32.5	287.5	416	577.5	353.5
Oyster Com	204	-38.5	-98	-87.5	841	564.5	320.5
Dermatoph	2	90	22	113	446	46	7.5
Lupine Lu	122	844	912	687.5	62	10	38.5
Cow's milk	261.5	497.5	421.5	563.5	237.5	75.5	-17.5
Epicoccum	1289	846.5	776.5	594	229.5	343	197
Co112 Co	483.5	1189	248.5	332.5	265	132.5	35
Cy5	261	145	93	61	1746.5	1192.5	1282.5

Parvalbumi	-55.5	2.5	-22	43.5	207	87.5	25.5
German co	5535.5	4146	4156.5	5130	10	-21	-46.5
Horse myog	149.5	489	716.5	500	1118	587	428
Bipolaris so	1436	813.5	985	576	214.5	167	126
Phl p 12 P	557	415.5	438.5	527	-34	-14.5	4
wheat extra	991	384.5	522	1097.5	197.5	160	93
Act d 5 Ac	99.5	442.5	260	472.5	201	210	171
Geotrichum	897.5	383	191	44	-13.5	26.5	-27.5
Spinach S	307	96	329.5	609.5	268.5	345.5	309
Pru p 7 Pr	128	269	185.5	216	-15.5	-25	-28.5
Act c 5 Ac	90	286	284	324.5	285.5	498	618.5
Tiger Nut (C	417.5	576.5	208.5	34	53	204	-40.5
Human IgG	2549.5	3271	3033	3112	381	434	312.5
Latex Patat	94.5	420	315	516.5	723.5	1016.5	795.5
Cladosporiu	2464	1293	1331	1182.5	22.5	-20	24
Cu09 Cu0	215.5	407.5	106.5	19	43.5	109.5	248.5
Eurotium a	1455	1101	978.5	1006	1636	806	993
Trypsin inhi	-45.5	55	-58.5	78	465.5	38	147
Mouse Epit	708.5	811.5	521	521.5	416.5	325.5	201.5
LTP from P.	247.5	31	74.5	314	814.5	1231	907.5
Carob Cer	-23	175	328.5	297	167	250.5	101
Dog IgM 1	149	283.5	201.5	634	590.5	826.5	991.5
Hen Egg wh	385	1080.5	263	298.5	328.5	200.5	357
Sycamore P	708	594	729	960.5	28.5	102.5	89.5
Poplar Whi	272	129	76	8	351	480	354.5
Oat extract	84	163.5	83	253	131	178	77.5
Parvalbumi	-53	14	31.5	91.5	498	592.5	522.5
Blomia Trop	1495.5	1095.5	1588	1065	343.5	317	238
Anisakis Tro	66.5	-36	-52	-179	703.5	778.5	121.5
Common B	134.5	285	225	506	110	120	42.5
Latex genui	23	300.5	-70	-8.5	131	217.5	88.5
Camel's mil	641	392.5	475	732.5	182.5	255	240
almond ext	1542	1115	1059	1430	132	93	45.5
Latex genui	77.5	36	-33	100.5	56	80	50.5
Hop pollen	1423	53	-105.5	-231.5	932	1065.5	1535.5
Aeromicrob	677.5	279	283	72.5	125.5	184.5	89.5
Gold kiwi (p	-95	105	83.5	-29	-155	-141.5	-284
Nettle	-6.5	-19	-2	170.5	65	84	20
Duck Egg w	162	710.5	186.5	95	149.5	119.5	23.5
German co	307.5	237.5	104	128	622	450.5	475.5
Ostrich egg	392.5	874	334.5	217.5	162	128.5	147
Aspergillus	640	495.5	390	267	84	-32.5	-10
Latex SOD	637.5	1068.5	566.5	418	254	168	105.5
Cy5	160	44.5	83.5	124	50	3.5	-49
Phl p 1 Ph	176.5	125	197	70.5	1036.5	781	612.5
Hen's egg g	398.5	254	248	231.5	113	160	48
Pru p 3 P	83.5	128.5	61.5	52.5	107	146.5	67
Dog Epithel	247	160	-7.5	-72.5	-16.5	3.5	-17.5
Mosquito	220	317.5	207	147	546.5	743	591

Cockroach,	1882	1636	1296	1066.5	479.5	554.5	361.5
Bet v 2 Be	1379	1459	1391.5	1099	276.5	345	182.5
Mugwort, C	311	895.5	-62.5	-57.5	121.5	145.5	122.5
Empty	12	127	-50.5	41	494	230	452.5
Pecan extra	82	-20	17	183	258	265	158.5
Lemon Cit	-628	-919.5	-898	-562.5	27.5	63	15.5
Helmeted G	162	580.5	452	425	480.5	323.5	210
Dog (?) IgA	-16.5	67.5	-34	-40.5	155.5	141	152.5
IgE one qua	91	-37	101	-42.5	1003	1249.5	1012
Aspergillus	865	600.5	787.5	481.5	170.5	691.5	209
Pru p Hevb	5	57.5	-81	-209.5	242.5	216.5	227
Hen's egg g	217.5	286	175	102	460.5	747	636
German co	2397.5	2163.5	1634.5	1784.5	200	103	238
Hen Egg yo	254	293.5	74	125.5	1464	994.5	663
Thermoacti	1544	1242.5	1190	1004	21	-35	89.5
Quail egg y	406.5	1044.5	100.5	-73.5	11.5	24	-85.5
Cat Epitheli	-6	5	-24.5	161	830.5	400.5	666
Cu13 Cu1	360	889	366.5	285	219.5	140.5	198
Japanese Sa	384.5	3	-63	-201	153.5	224	113
Cow's milk	127	1390.5	212.5	88	1812	1360	1155
cocomilk ex	299.5	524.5	437.5	438.5	168.5	191	178.5
Cy5	298	169.5	4.5	152	561	-11.5	48
Lobster H	1304.5	1542	1553.5	1618	720	803	745.5
cu04 cu04	97.5	-44.5	-58.5	144.5	150.5	248.5	77.5
Alternaria g	531.5	833.5	661.5	618.5	4333.5	4177.5	3887.5
Cashew ext	667	605.5	579	811.5	2	67.5	64.5
Der f 2 De	296.5	252.5	278.5	340.5	105	99.5	168
cuN cuN	394	569.5	584.5	1014.5	44	49.5	12
basil extrac	93.5	28	184	138.5	105.5	186.5	188
Storage mit	59	249.5	160	699.5	312	643	354.5
Human IgG	1096	920	1178.5	1086	88	154	129.5
IgE half dilu	175	566.5	349.5	319	164	16.5	29
DogIgG 1m	1393	1901	2303.5	2148.5	233.5	417	327.5
Cockroach	1325.5	238	166.5	-61.5	222	158	122
Horse Epith	93	140	-64.5	-84.5	250.5	328.5	121
Der f 1 De	573.5	361	592	482	312	409	316.5
Leek All p	620	464	203.5	-36	32.5	507.5	122
carrot extra	39	394.5	-39	200.5	142.5	263	186
SiMVM Si	890.5	1162	557	603.5	115	152.5	67
Soybean AL	-29	597.5	-97.5	-132	362	130.5	112
Duck Egg yo	125	37.5	-44.5	9	2826	139.5	25.5
Act d 1 A	56	967.5	78	-34	274.5	238	194.5
Alder europ	916	491.5	312.5	411.5	-60.5	-162	-219
Trout Onc	1.5	-38.5	-47.5	76.5	74	97	66
Alternaria E	41.5	362.5	693.5	798.5	134	189	257.5
empty	-37.5	-6.5	-0.5	50	112.5	104	124
Act c chitin	124	168.5	41.5	184	283	4182.5	161.5
Sycamore p	424	268.5	28.5	41	9.5	-35.5	-28
wool sheep	374	0.5	-76.5	-117.5	99.5	136.5	180

LTP - Short	12	5.5	-11.5	2	289	188.5	158
Cow's milk	409.5	623	521	686	230	165.5	72
Goose Egg	244.5	914.5	54.5	53.5	-371	-345.5	-520.5
European H	136	250.5	44	-51	804.5	1223	889
Act c 10 A	28.5	385	-17	-155.5	10.5	-27	20
CuX CuX	1066	1332	903	466.5	99	67.5	42.5
Alt a 1 1m	217.5	303	209	114.5	-57.5	-32.5	-79.5
Human's co	188	227	194	434	35.5	-28	-5.5
Parietaria P	131.5	278	63.5	90.5	3.5	90.5	12.5
Latex Heve	678.5	849.5	442	469	231	254.5	180
Rabbit Dan	74.5	6	311	172	57	-30	23.5
Cow's milk	102	166.5	112.5	186.5	30.5	536.5	55.5
Bet v 1a B	430	538.5	326.5	387	-120	-38.5	-240
Ash allergo	-0.5	-28.5	-55.5	-74	-19	83	1
Ber e 1 Be	-6.5	59.5	317	257	78.5	42.5	63
Birch allerg	95	6.5	54.5	71	589	368	480
Der p 1 D	288.5	196.5	180	348.5	372	251.5	164.5
empty	398.5	27.5	60	-0.5	1010.5	206.5	163.5
DogIgG 10	5855	6651	7428.5	7617.5	419.5	494	295.5
Gly m 4 0	115.5	81	11	214	105	73	-27
Hen's egg g	288.5	456.5	419	136	136.5	163.5	99.5
Cow (beef e	141.5	176.5	109.5	-26.5	117	60.5	105.5
Hazel pollen	252	261	181.5	246	907.5	633	923
Salsola PME	3096.5	2571	983	2376.5	-18	-27	-15
Cy5	180.5	108	252	272	186.5	90.5	156
Cu03 Cu0	381	280.5	415	557.5	313.5	601.5	267
Nettle Urtic	255.5	148	25.5	53	268	306.5	208.5
Durum Wh	362	442	696.5	751.5	61.5	39	-9
Rat epitheli	1379	651.5	429.5	508	284.5	332.5	260.5
Mosquito	371	340.5	134	263	-54	-120	-163.5
Corn Seed (	681.5	953	823	1755	103.5	50	127.5
Blood Amar	153.5	273.5	-135.5	-75.5	-18.5	-0.5	-19
Act d 10 A	51	74.5	13	292.5	550.5	692	454
HRP as CCD	5987.5	4106.5	52	210.5	50.5	72	44
parsley extr	188.5	592	20.5	152	221.5	197	128
Lobster Nor	888	993	494	1127.5	281	114	86.5
Annual Mer	838.5	638	527.5	747.5	198.5	226	199.5
Human's m	1364	591.5	455	555.5	44	28	-23.5
Cat allergo	3.5	-45	-32	50	9	15	1.5
Parvalbumi	67.5	238.5	60.5	1943	303	332.5	242
German co	6867	5884	5290	6021	48	27.5	-14.5
Empty	41.5	10	22.5	218	-1	13.5	-12.5
Cu08 Cu0	268.5	314	299.5	278.5	542.5	431.5	340.5
Act d 2 A	256	209.5	86	290.5	75.5	98	66
Camel's mil	167.5	116	81	-50	-149	-191	-257
Alpaca's mi	1381.5	1584.5	859	1771.5	330.5	266.5	197.5
Act c 1	249.5	299.5	190	161	31	78	-9
Cow's blood	575.5	669.5	592.5	669.5	21.5	43.5	-13.5
peas extrac	-3.5	104	-89	2	83.5	73	56

Der p 2 D	1148	1448.5	567	969.5	29	53.5	104.5
Avocado P	143.5	401	-136	669.5	-30.5	-19	-16
Cy5	185.5	254.5	181	679	129.5	84.5	88.5
Bermuda gr	514.5	654.5	218	551.5	16.5	23	-5
Empty	-14.5	-3.5	-148	31.5	38.5	-59.5	10
Lepidoglyph	31494.5	32343.5	32352	34021.5	76.5	100.5	99.5
Hen's egg g	141.5	155.5	215.5	229.5	23	21	-24
Cow's milk	39.5	76.5	98	-9.5	274.5	281.5	297
Cow's milk	242	401.5	715	405	-4	13	-56
Grape Vit	-668	-773	-678.5	-963.5	342	305.5	324
Goose Egg	562	481.5	287.5	308.5	144.5	97.5	20.5
Mouse Lipo	16	57.5	111	120	253	216	129.5
wheat extra	241	332	195.5	287.5	384.5	321.5	242
sun flower	2893	2562.5	1733.5	1191	28	63.5	64
Act d 11 A	103.5	128	171.5	102.5	38.5	4.5	23.5
Act c 11 A	91.5	61.5	74	67	203.5	150	108
Rat Lipocali	181	100.5	98	166	246	212.5	156.5
Rhodococc	4316.5	3636	2630.5	2269	83	92	60.5
harriot bea	407.5	353	128.5	501	551	605.5	707.5
Cy5	338	477	132	76	5.5	10.5	-3.5
Peafowl eg	74	147.5	-16.5	-40	-17	18.5	26
rye extract	-39.5	-70.5	22.5	177	-91	-87.5	-235
Oyster Mus	34.5	77.5	-47.5	-53	86.5	76.5	50.5
Flea Ctenoc	53.5	68.5	-49.5	68	30.5	22.5	-33.5
Orange Ci	-529	-804.5	-895	-703	15.5	8.5	-4.5
Brazil nut e	1467.5	1585.5	2026	2981	116	96	59
Cow's milk	123.5	155.5	157	902	331.5	243	94.5
Green kiwi	15.5	33	132	91.5	479.5	447.5	516
Melon Cu	-63.5	-167.5	-134	-109	65.5	53.5	20.5
Empty	42	82.5	24	80	4430	3011.5	3532.5
Poplar Whi	672	913.5	45	222.5	207.5	184.5	166
Dog's Lipoc	245	259.5	358	406.5	-13	2	-6.5
Wheat Glia	434.5	646	1343.5	1438.5	49.5	57	54.5
Timothy po	75.5	51	80.5	288	80	61.5	52.5
Human albu	210.5	191.5	75	102.5	39	27.5	26
Phl p 6 Ph	1701.5	1681	1256	1421	342.5	305.5	259
Ostrich egg	204	124.5	100	79.5	608	516.5	618.5
Horse Albu	66.5	80	62	167.5	613	563.5	459.5
Mite genuin	1007.5	888	728.5	670	375	337	362
Cy3 1mg/	1323	1160.5	1203.5	921	41	52.5	63
Cypress, Ar	-43	-51.5	44	196	203.5	223.5	113
IgE Neat	316	257	707	776	6.5	8.5	-39.5
Papain Ca	437	351.5	258	186.5	1165	1103.5	1166.5
cod extract	58	38	12.5	30.5	114.5	132	14.5
turkey egg	469.5	467.5	290	93	89	94.5	40.5
Microbacte	1402	1277	1991.5	1536.5	54	63.5	145
banana ext	63.5	96.5	56.5	-7.5	293	271.5	103
turkey mea	306.5	271	198	315.5	168	163	42
red pepper	-25	-30.5	7	52.5	302.5	305.5	268.5

Fusarium so	23	44	127	-153	232.5	216.5	141
Kamut Tri	524	538.5	438	828	127	122.5	90.5
Latex Profil	309	267	103.5	569	592	575	659.5
pine kernel	312.5	325	225.5	377	189.5	211	108.5
Birch Europ	407.5	396.5	290.5	158	944.5	950	904
Culicoides C	306	292	43.5	97	-240.5	-237	-311
Squid Lol	366.5	350.5	215.5	209.5	2383.5	2340.5	1609
Sycamore G	146.5	134.5	77.5	54	13	22	54
Octopus Co	9.5	16	33	201.5	79.5	76	82
Olive pollen	798.5	783	388.5	848.5	964.5	972	575.5
Dog (?) IgE	0	-10.5	-10.5	297.5	255	258.5	258.5
Apple Bet v	50	63.5	21	209	60.5	62	13.5
Cow's milk	88	94	19	-113	12.5	11.5	-16.5
Sheep's epi	-14.5	-12	-74.5	-38.5	109	111	78.5
soy bean le	165.5	174.5	261.5	237.5	119	111.5	84.5
Soybean AL	-23	-19.5	-223.5	-224	234	236.5	220
Sorrel red R	-16.5	-14.5	-69	17.5	32.5	33	42
Hazelnut 11	251	249.5	377	460	550.5	551.5	440.5
Buckwheat	163	165	60.5	457	408	407	311.5
Guinea pig	66	66.5	-4.5	-60	239.5	239	159
Dermatoph	-65.5	-37.5	26	123.5	41.5	41.5	4.5

F STEAMEDV SERUM AV DRY AV V SOAKED AV STEAMED

414.5 154.5 279 225.5 297

136.5	1045.5	330	406.5	271.5
448	2631	2189.5	1967.5	3237
914.5	1161	419.5	391.5	520
2550	54	110.5	115	68.5
53	9114	2928	3823.5	4344
649	953.5	-17.5	-1.5	3.5
46	5.5	171.5	90.5	91.5
17.5	2465.5	91	121.5	65
287	140.5	267	209	68
130	-193.5	-120	-190.5	-159
48	115	236.5	145	135
90.5	-127	-81	-131	-76
353.5	-199	-133.5	-155.5	-128.5
1454.5	58	215	151.5	173.5
129	153.5	117.5	-7	99.5
46	162.5	21.5	62.5	29.5
135	169.5	117	110	109
51.5	190	120	149.5	117.5
49.5	945	572	739	626.5
3208.5	625	710	613	508
70	282	443.5	397.5	450
113	3714.5	35	153.5	113.5
21	1903.5	1103	655.5	840.5
2094	2683	3586.5	4515	2210.5
254.5	98.5	33	38	36.5
115	2527	111.5	191	203
70.5	142	160	186	114

322	1259.5	630	2506.5	644
106	1613.5	717	411	1077.5
333.5	3443.5	1661.5	1813	2083.5
1199.5	2379	863.5	1202	1201
115	865	308	55	145.5
5611	3918.5	2366	2056.5	1707
125.5	-296.5	-206.5	-59	-263.5
248.5	17.5	77.5	64	107
157.5	842.5	734.5	852.5	832.5
10.5	108	516.5	252.5	351.5
94	57	229.5	132	86
175	27	102	140.5	153.5
64	5.5	30.5	9	17.5
216	446	545	367.5	412.5
219.5	205	170	165.5	144.5
178.5	196.5	236	174	213.5
98.5	-1.5	38	17.5	34.5
58	4	256	198.5	147.5
66.5	123.5	160	107.5	150.5

150.5	811.5	132	160.5	125.5
123.5	397	185.5	192	103.5
338	-50.5	5	14	-13
1268.5	16	86	28	42.5
93	1218	671	874.5	713.5
302	128.5	99.5	79	106
249.5	670	376	232	380
473	307	-34.5	-6.5	-11.5
-12	-24.5	16	0	16.5
68	38.5	188.5	69	143
-27	615	332.5	376.5	234.5
82	1681.5	378.5	287	358
6	-15	72.5	84	25.5
55	36.5	101.5	47.5	33
26.5	64.5	28	47.5	24.5
363	942.5	746	499	587.5
192.5	165	33	60.5	64.5
-23	492.5	45.5	218.5	72.5
197	898.5	1326.5	1078	1203.5
21.5	392	759	420	611.5
710.5	1677	245.5	430	124
705.5	338.5	223.5	296.5	298
609.5	932.5	613.5	635	565.5
476.5	147.5	74	40.5	68
196.5	29.5	68	7.5	20
46	1401	651	1040	830.5
328.5	1115	408	850	401.5
2321	62.5	141.5	87.5	112.5
68.5	122	66.5	30	29
80	7569	2895.5	4152	2713

278.5	1888	567.5	463.5	897
5	446	233.5	219.5	168.5
251	2291	897	847.5	964.5
1296	65.5	102.5	28.5	56
36	308.5	150	176	242.5
446	1060	299	208	192.5
6675.5	35.5	184	130	159.5
1408	147.5	195	148	218
963	316	156	136	176.5
1637	884.5	333	333.5	196.5
7.5	46	171.5	109.5	160.5
470.5	-115	2	-85	-16.5
77	488	637.5	504.5	690.5
79.5	300.5	140.5	143.5	236.5
584	67	-13	28.5	17.5
266.5	-7.5	27.5	53.5	21
155	278.5	150.5	162	185.5
78	243	150	178.5	170.5

68.5	154.5	198.5	152.5	131.5
168.5	186	290.5	205.5	198
46.5	792	28.5	-8	4
168	-17	8.5	3	1
1261	34.5	116.5	77	62.5
584	19.5	41.5	64	32
1513.5	959	1135.5	1087	968
118.5	96	174	178	103
146	1672	88.5	76.5	85
1113.5	-19.5	87.5	80	51
199	127	224	161.5	171
96.5	-17.5	30.5	47.5	7
-4.5	1906	889	972	900
135.5	481.5	170	110.5	168
1265	117	175.5	99.5	180
1276	138	389	187	149
235.5	107	157.5	109	130.5
1017	184	62	131	102
3851.5	103.5	81.5	69	86.5
1	6.5	23	269.5	78.5
1937.5	22	-22	-10.5	-0.5
135.5	262	187	200	135.5
57.5	80.5	217	111	145.5
373	28.5	122	104	80
233.5	216	374	208.5	181
498	-17.5	3.5	-16.5	-5
340.5	16.5	58.5	13.5	17.5
251	97	121	81.5	118
59.5	4.5	38.5	16.5	15
944	70	53	51	-1
66.5	65	136	147.5	80

136	0.5	54.5	6.5	29
1517.5	125.5	224	201	355.5
574.5	-214.5	-173	-218	-122
24.5	658.5	595.5	438	626
183.5	138	83	141	19
1021	1098	396	109.5	216
348.5	237.5	140.5	140	92
-15.5	876	190.5	69	107
268	1503	228	307.5	238.5
134	73	6	51.5	36
95.5	224.5	378	234	188.5
1588	1394.5	1328.5	1271.5	1048.5
298.5	572	310	226.5	201.5
200.5	694.5	128	79.5	80
839.5	3842	1585.5	1897.5	1440
33.5	16.5	4.5	137	93.5
53.5	304.5	174.5	185.5	207

359.5	259	374.5	269	445.5
81.5	243.5	170.5	186.5	123
341	94	189	100	195.5
310	386.5	772.5	1040.5	407.5
102	64.5	44.5	287.5	25
63.5	377.5	299	246.5	189.5
511	815.5	1257	817.5	967
1750.5	2281	2213	2645	1837
222.5	3069	2803	2766	2692
215	86.5	176	109.5	168
488	226.5	346	183.5	233
128.5	-8.5	21.5	24	47
-43	278.5	350	775.5	302
316	1385	267.5	302.5	223.5
4.5	52	113	44	119.5
68	403	563	419.5	370.5
279.5	124	229.5	111	136
217	39.5	85	-18	46.5
162.5	1150	270.5	203	771
178	-32	22	5	54
156.5	7569.5	5629.5	4991.5	4589.5
82.5	917	537	718.5	237
50.5	619	179	71	194
429	135.5	224.5	232	218
66	313	455.5	376.5	446
-8.5	-21	42	13.5	52.5
428	29	39	145	30.5
378.5	269	416	595.5	163.5
155	304.5	227.5	171.5	233.5
58.5	49.5	71.5	28.5	37
73.5	2333	1708.5	1653.5	1718.5
105.5	589.5	371	355.5	359

81.5	44	205.5	25.5	22.5
122	-133	-48	-143.5	-85.5
1229.5	1164	983	979	989
241	77	108	64.5	329
32.5	1824.5	1539	1262.5	1893.5
74	67	119	57	138
349.5	100	204	202.5	193.5
285.5	173	153.5	175	188
459.5	304.5	375.5	225	302.5
66.5	100	18	18.5	36.5

18 55 14 17.5 43.5

19 679 750.5 216.5 418

31.5	-22	12	2.5	-12.5
284.5	7.5	38	-11.5	-5
103.5	41.5	120	106.5	129.5
1002	740	348	259	306.5
112.5	320	192	238	195.5
42.5	163.5	126	151	196
778	1732.5	1466.5	1444.5	1577.5
184	1	-51	27	393
-30.5	229.5	134.5	184.5	174
55	590	132	195	226.5
128	164.5	186	153	234.5
137.5	104	180	152	142.5
301	25.5	21.5	11.5	-10
110.5	-5.5	39	30	8
535.5	1927	1328.5	962	1326.5
22.5	342.5	289	307.5	263.5
262.5	64	95.5	63.5	73
4137	23.5	101.5	99.5	29
11	404.5	152	192.5	125
155.5	272.5	76	440	-15
1001	264.5	336	248.5	217.5
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232.5	176	210.5	198.5	203.5
660	106	504	571.5	665
240	-39.5	152	7.5	22.5
843	274	250	247	267.5
135.5	-10	-12.5	205.5	-7
132	141.5	203	192	134
396.5	389.5	274	220	217
187	166	265	261.5	264
466.5	462	267	151.5	161
337	1487	991	818	1227.5
593.5	157.5	182	68.5	169.5
148	-8	25	47	18
96	2366	2219	2038.5	2227
257.5	6	12	24	-11

199	994	877.5	1573	588.5
39	-242	-198.5	-134	-168.5
1055.5	99	61	139.5	22
129.5	138.5	191.5	135.5	228
-253.5	110	89.5	58.5	105.5
54	454.5	423.5	409.5	436.5
93	208.5	9	67	23.5
406	231.5	282.5	217.5	282
177	86	122.5	81.5	151.5
6	-19.5	-8.5	85.5	-6.5
201.5	28.5	119	21.5	18.5
10	131.5	105	45.5	52.5
255.5	25	124.5	92	41
82.5	-12.5	3	6.5	9.5
111	398.5	246.5	404.5	218
21.5	24.5	-9.5	-26.5	-11.5

678.5	360	456.5	225	419
482	81	85.5	42	69
196	24	-9	-12.5	-18
99	-58.5	-39.5	-43.5	-50
339.5	33	13.5	-1.5	-12.5
149	590.5	1315.5	512.5	584.5
30.5	177.5	77	79.5	56.5
199.5	229	180	120.5	270
47.5	1	39.5	48.5	-1
884.5	160	110.5	69.5	68
106	-30.5	76.5	-16	-8
203.5	68	41.5	11.5	52
31	139.5	111	195.5	115
487	241	232.5	124.5	197.5
591	116	289	120.5	125
-30	-1	26	4.5	12.5
5	308.5	269	221.5	194.5
-1	794	671.5	637	616
114	-47.5	27.5	-79	-27.5
62.5	117.5	53	68.5	8
1592.5	123	201	79.5	123.5
661	454.5	239.5	113	295
88	0.5	-11.5	5.5	25
647	321.5	46.5	21	24
24	806.5	11.5	37	-5.5
3829.5	326.5	52.5	57.5	60
45.5	391.5	124.5	125.5	182
293.5	139.5	194	110	222
41	30	59	5	41
57	72	87.5	76	74
277.5	5	18.5	-15.5	9
144	95.5	130.5	97.5	91
-9	68	121	20	24.5

319.5	21.5	7.5	-35	61
175.5	569	21	-13	8
320.5	2460.5	85.5	94	16
332	50	-10.5	1	-24
160	41.5	-5	35	27
240.5	146	183	102.5	125
135.5	180.5	302	160	149.5
199.5	-28	30	17.5	30.5
153.5	150	106.5	63.5	119
168.5	9.5	48.5	7.5	22.5
-136	171	569.5	149	345.5
20.5	-22	-9	-4.5	26
198.5	188.5	267.5	175.5	175.5
330	4	34	50.5	65.5
214	552	203.5	183	222
-18.5	144	173.5	192	141
133	1315.5	1460	958.5	2424
214	342	77.5	89.5	71
94	3251.5	1387.5	2457	1094.5
-293	301.5	56	40.5	75
1102	451	596.5	527.5	509.5
114	332.5	130	7	96
12.5	704	471	971.5	764
-80.5	242.5	-8.5	-30.5	-38.5
-3	342.5	274.5	297.5	336
60	108	34	117.5	44.5
365	29.5	-17	160	-47.5
42.5	519	401	271.5	287.5
65.5	58	-12	16.5	31
2	366.5	56.5	18.5	84
10.5	880.5	196	135	177
75.5	75	57.5	94	32
619.5	101	84	82.5	72.5
194	298	186	143.5	348
740	273	217	91.5	205
393	33	4	-8	44
-23.5	487	557.5	533	355.5
149	351.5	211.5	263	112
144	-10.5	38	-15.5	-30.5
38.5	-16.5	49.5	22.5	7.5
-10.5	227	86	252	71
137	247	51	-16	33.5
857	319	223	217.5	281
290	47	-13.5	-36	26.5
46	44	54.5	22	25
401.5	140.5	33.5	87	521
-160	174.5	152	77	134
116.5	86.5	84.5	80	117.5
-11.5	218.5	209.5	274	136.5

393.5	-37.5	-21	26	-14
42.5	-18	-5	-12.5	51.5
184	60	84	608	55
33	400.5	467	363	436.5
166.5	710	666	648.5	605.5
44	70.5	73.5	88.5	43
51.5	36.5	41	259	32
237	161.5	204	144	125
52	175.5	117	10.5	66
-32	354	386	345	633.5
364	600.5	500	444.5	399.5
89	549.5	414.5	431.5	332
-207	907.5	985	635.5	1536
147	395.5	248	223.5	137
54	80	129	92	79.5
4	77.5	63.5	10	53
56.5	51	9	-7.5	-18.5
43.5	806.5	891	570	613
226	280.5	244.5	185.5	231.5
21	210.5	114	424.5	118.5
-8.5	479.5	487	349	564
2.5	75.5	103.5	85.5	101.5
67	231.5	98	66.5	101
45	4.5	-15.5	22	3
285.5	-13	5	45	5
-22	277.5	208	117	132
159.5	135	160.5	124	366
18.5	15.5	37	32.5	285.5
199	127.5	110	67	71
336	26	46.5	28	25
322.5	58.5	64	52	43
7	181	147.5	108.5	146
161	434.5	319.5	337	209.5
217.5	294.5	261.5	224.5	183
69.5	3365.5	3844	2497.5	2749.5
373	91.5	88	168.5	65.5
4.5	95.5	88	15.5	96.5
5	-20	-22.5	-18	58
-114.5	38	11.5	10.5	25.5
78	114	108.5	64.5	117
-4.5	499	445.5	415.5	547
6	56.5	49.5	58.5	27
46	1128.5	977	700	775.5
224	266	311	261	291
405	149	128	101	102
48.5	565.5	592	485	406
2464	212.5	235.5	137	147
188.5	230	183	77	172.5
-3	12	2.5	-13	4.5

130.5	83	91	82	93.5
16.5	129.5	125.5	8.5	124.5
36	3485.5	3357	3078.5	3537.5
562	-18.5	-16	2	29.5
638.5	138	125.5	144	151
522	6	11.5	62	11
227	383	396	311	368.5
35	317.5	278.5	276	186
195	-15	-4.5	30.5	-10
62.5	93.5	85.5	54	129.5
772	212	227.5	410	183.5
61.5	62	50.5	38.5	63
46.5	107.5	94.5	79	77

55	327	341	330	274
166	220.5	230	118.5	308
139.5	427.5	403	522	285
413.5	122.5	133	33	165.5
199	67.5	78	45.5	54.5
145.5	194.5	175.5	214	258
557	615.5	602.5	442.5	446.5
69	161	151.5	55	197.5
898.5	550.5	549.5	473.5	445
-245	62	54	51.5	95.5
2341	-26	-31	167.5	61.5
129	158.5	167	153.5	129
61.5	70.5	64.5	163	111.5
828.5	65.5	70.5	6.5	79.5
229.5	266	261	2833	182.5
38	14	17	4	18
19	4	5.5	1	17
66.5	98.5	100.5	43.5	79
128	-7	-8	2	-14
150.5	206.5	205.5	201.5	208
50.5	30	32	10.5	16.5
350	49	50	87.5	18
349.5	-2.5	-18.5	19	18
212	583	973	668.5	621.5
11.5	-32.5	141.5	58.5	110